Cytoprotective Roles of Oat Avenathramides Against Inflammation and Cellular Stress

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ABSTRACT

Natural polyphenols have been considered as promising anti-aging compounds not only for their antioxidant activity, many of them also function as signaling mediators that modulate cellular pathways involved in cytoprotection. Avenanthramides (Avns), a group of polyphenols found exclusively in oats, are natural antioxidants associated with human health promotion. In this study, avenanthramide A, B and C (Avn A, B and C), the three most abundant Avns in oats, were identified and quantified from oat phenolic-rich extracts of ten oat varieties. In addition, *in vitro* antioxidant activities of oat extracts and Avn A, B and C were evaluated. It was found that Avn C had the highest *in vitro* antioxidant activity among the three Avns. To investigate the cytoprotective activity of Avn C, normal human skin fibroblasts (2DD) were treated with Avn C followed by exposure to extracellular stress and its ability to reduce cellular damage was determined. Pre-treatment of cells with Avn C reduced hydrogen peroxide (H_2O_2)-induced oxidative stress significantly as demonstrated by decreased intracellular free radical levels and antioxidant gene transcripts. Avn C pre-treatment also resulted in decreased levels of gene transcripts encoding pro-inflammatory cytokines in response to H_2O_2 or tumor necrosis factor α (TNF-α) stimulation. This reduction in cytokine gene transcription occurred concomitantly with reduced phosphorylated nuclear factor-κB (NF-κB) p65, indicating reduced pro-inflammatory response. To better understand the mechanisms of actions, the impact of Avn C on cellular signaling pathways was investigated on Avn C-treated 2DD cells without exposure to stress. We found that Avn C induced heme oxygenase-1 (HO-1) expression through increased DNA-Nrf2 binding activity. Also, it reduced basal levels of pro-inflammatory cytokines through decreased DNA-NF-κB binding activity. Those mechanism pathways are independent of free radical scavenging and strongly associated with oxidation, inflammation and the aging process. Moreover, anti-proliferative effect of Avn C on 2DD cells was observed via mechanisms independent of autophagy activation. Collectively, our findings suggest that Avn C protects normal human skin fibroblasts against oxidative stress and inflammatory response through Nrf2/HO-1 activation and NF-κB inhibition and further imply that Avn C may be a potential nutraceutical for health promotion and disease prevention.

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LIST OF ABBREVIATIONS

2DD	Normal human skin fibroblasts
A	Absorbance
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AKT	Protein kinase B
AMPK	5' AMP-activated protein kinase
ANOVA	Analysis of variance
ARE	Antioxidant response element
Avn/Avns	Avenanthramide/Avenanthramides
Avn A	Avenanthramide A
Avn B	Avenanthramide B
Avn C	Avenanthramide C
bp	Base pair
BSA	Bovine serum albumin
°C	Degree Celsius
CAT	Catalase
Cat #	Catalog number
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
cm	Centimeters
CO	Carbon monoxide
CO_2	Carbon dioxide
COX-2	Cyclooxygenase 2
Ct	Cycle threshold
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picryl-hydrazyl-hydrate
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGCG	Epigallocatechin gallate
FBS	Fetal bovine serum
FC	Folin-Ciocalteau
Fe^{2+}	Ferrous
g	Grams
GAE	Gallic acid equivalent
GPx	Glutathione peroxidase
GSH	Glutathione
GSS	Glutathione synthetase
h	Hours
HO	Heme oxygenase
H_2O_2	Hydrogen peroxide
HPLC	High performance liquid chromatography
IC_{50}	Concentration for 50% radical inhibition
IκB	Inhibitor of kappa B
IKK	Inhibitor of kappa B kinase
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
IL-8	Interleukin 8
Keap1	Kelch-like-ECH-associated protein 1
LC3	Light chain 3
M	Molar
μg	Micrograms
μL	Microliters

μm	Micrometers
μM	Micromolar
MAPK	Mitogen-activated protein kinase
mAU	Milli-absorbance units
mg	Milligrams
min	Minutes
mL	Milliliters
mm	Millimeters
mM	Millimolar
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor □-light-chain-enhancer of activated B cells
ng	Nanograms
nm	Nanometers
nM	Nanomolar
NQO1	NAD(P)H dehydrogenase [quinone] 1
Nrf2	NF-erythroid 2-related factor 2
O_2	Oxygen
$O_2\bullet^-$	Superoxide anions
$^\bullet OH$	Hydroxyl radical
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline with 0.05% Tween® 20
PCR	Polymerase chain reaction
PDA	Photodiode array
PI3K	Phosphoinositide 3-kinase
p-p65	Phosphorylated NF-κB p65
ppm	Parts per million

qPCR	Quantitative polymerase chain reaction
r	Correlation coefficient
rcf	Relative centrifugal field
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
rRNA	Ribosomal RNA
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
s	Seconds
SDS	Sodium dodecyl sulfate
SIRT1	Sirtuin 1
SOD	Superoxide dismutase
TNFα	Tumor necrosis factor □
UV	Ultraviolet

1. INTRODUCTION

1.1 Overview

Oats (*Avena sativa* L.) have many scientifically verified health-promoting effects, making them one of the best grains for human consumption (Butt *et al.*, 2008; Martínez-Villaluenga and Peñas, 2017). The majority of the health benefits of oats are attributed to the cholesterol-lowering potential of β-glucan (Othman *et al.*, 2011); however, several phytochemicals have also been found and recognized for their positive impacts on human health, which include phenolic compounds (Peterson, 2001). Since most phenolics are found in the bran layer of oat grains, oats could be a significant source of dietary antioxidants as they are normally consumed as whole-grain cereal (Peterson, 2001; Ryan *et al.*, 2011). Major compounds that exhibit antioxidant activity in oats are vitamin E (tocols), phytic acid, phenolic acids, and avenanthramides (Avns) (Collins *et al.*, 1991; Peterson, 2001). Avns have received increased attention in recent years because they are a group of unique phenolic compounds found exclusively in oats and exhibit high antioxidant activity (Collins, 1989). Avns are conjugates of a phenylpropanoid (*p*-coumaric, ferulic, or caffeic acid) with anthranilic acid or 5-hydroxy anthranilic acid (Collins, 1989). The majority of Avns can be found in oat groats, with the highest concentration in the bran. More than 20 different forms of Avns have been identified and the three most abundant Avns in oats have been determined and named as avenanthramide A (Avn A), avenanthramide B (Avn B) and avenanthramide C (Avn C) (Peterson, 2001).

Avns were identified in oat groats and hullsby Collins (Collins, 1989). Subsequently, Dimberg and colleagues measured their antioxidant activity by the inhibition of oxygen consumption in a linoleic acid system and reported that their activity was 10-30 times higher than those of caffeic acid, ferulic acid and vanillin isolated from oats (Dimberg *et al.*, 1993). Since then, a number of studies have been focused on investigating health-promoting effect of Avns or Avn-enriched oat extracts. It has been found that Avns showed strong antioxidant activity? both *in vitro* and *in vivo* with Avn C being the highest (Dimberg *et al.*, 1993; Peterson *et al.*, 2002; Ji *et al.*, 2003; Lee-Manion *et al.*, 2009; Yang *et al.*, 2014). Moreover, several studies have indicated that both oat phenolic-rich extract and pure Avns had anti-inflammatory activity by reducing the expression of pro-inflammatory cytokines and repressing nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) function (Guo *et al.*, 2008; Sur *et al.*, 2008; Chu *et al.*, 2013; Yang *et al.*, 2014). In addition, Avn C was also reported to inhibit the proliferation of rat vascular

smooth muscle cells through modulation of cell cycle, indicating the potential health benefit of oat consumption in the prevention of coronary heart disease (Nie *et al*, 2006a; 2006b). Notably, the structures of Avns are very similar to a synthetic anti-allergic drug called Tranilast, which also has been found to have anti-proliferative effect on rat vascular smooth muscle cells (Tanaka *et al.*, 1994). Recently, the anti-proliferative effects of Avns have also been examined on several cancerous cell lines and found that Avn C was able to decrease the proliferative rates of colonic cancer cell lines, including Caco-2, HT29, LS174T, HCT116 (Guo *et al.*, 2010) and MDA-MB-231 breast cancer (Hastings and Kenealey, 2017).

Most natural antioxidants found in our diet exhibit few side effects in humans due to their rapid metabolic rate (Chow *et al.*, 2003; Manach *et al.*, 2005); therefore, investigation of preventive effect of Avns may bring some new insights for nutraceutical development. Although several studies have shown that Avn C had strong antioxidant property through its free radical scavenging *in vitro* (Dimberg *et al.*, 1993; Peterson *et al.*, 2002; Lee-Manion *et al.*, 2009; Yang *et al.*, 2014), no study has addressed the protective effect of Avn C against cellular oxidative stress. Therefore, investigation of the intracellular antioxidant effect of Avn C using a human cell model could increase the understanding of the efficacy of Avn C. Additionally, previous studies have focused on the therapeutic potential of Avns in disease or mouse models (Guo *et al.*, 2008; Sur *et al.*, 2008; Chu *et al.*, 2013; Yang *et al.*, 2014); however, there is limited evidence on the protective effect of Avn C against pro-inflammatory signalling in normal human cells. Also, the mechanisms of health-promoting effect of Avn C are still poorly understood. More importantly, disease prevention is usually achieved by multiple cytoprotective effects working together such as antioxidant, anti-inflammatory, antigenotoxic and antiproliferation through different or overlapping cellular pathways, resulting in an overall promoted health and increased lifespan (De la Fuente and Miquel, 2009; Xia *et al.*, 2016). Therefore, exploration of multiple health benefits of Avn C and mechanism investigation may provide a comprehensive understanding of its function.

1.2 Hypothesis

Based on previous observations and the polyphenolic nature of Avn C, the central hypothesis of this research is that Avn C not only has potential antioxidant activity, it can also

activate signalling cascades that regulate gene expression involved in cytoprotection and regulates proliferative rates of normal human cells and cancer cells.

1.3 Objectives

In addressing the hypothesis of this research, the following objectives were studied: (1) extract, identify and quantify Avn A, B and C from oat seeds of ten varieties; (2) evaluate antioxidant activities of oat phenolic-rich extracts and Avn A, B & C employing ABTS and DPPH free radical scavenging systems; (3) investigate the protective effects of Avn C against hydrogen peroxide (H_2O_2)-induced cellular damage including oxidative stress, DNA damage and inflammatory responses in normal human skin fibroblasts; (4) investigate the impact of Avn C on gene regulation involved in inflammation and cryoprotection; and (5) determine the impact of Avn C on the proliferation of human normal skin cells and cancer cells.

2. LITERATURE REVIEW

2.1 Oats

2.1.1 Oat grains

Oat (*Avena sativa* L.) is an important cereal crop in the developing world and is known as common covered white oat (Butt *et al.*, 2008). Oat is an annual crop, and is predominantly grown in American and European countries, mainly Russia, Canada and United States of America. Compared to wheat, oat requires lesser nutrients but more moisture to cultivate (Ahmad *et al.* 2010). It was used mostly for animal feeding until the emerging awareness of oats as a healthy food for human consumption in the mid-1980s. Since then, the use of oat as animal feed has declined steadily and has become more popular for human nutrition (Butt *et al.,* 2008). Because oats are gluten-free cereals and not suitable for bread making, they are often served as porridge, biscuits, cookies, or breakfast cereals made from crushed or rolled oats (Butt *et al.*, 2008). The major components of an oat grain are the hull, bran, endosperm and germ (embryo) as shown in Figure 2.1. Hull of an oat grain is about 25-30% of the seed. After removal of hulls from oat grains, the rest of oat seeds (whole oat groats) are further processed for human consumption or animal feed production (Rasane *et al.*, 2015). Whole oat groats contain high amount of valuable nutrients such as soluble fibers, proteins, unsaturated fatty acids, vitamins, minerals, and phytochemicals (Rasane *et al.*, 2015). Consumption of oat products has been associated with the prevention of chronic diseases, such as cardiovascular diseases, diabetes and gastrointestinal disorders; therefore, increasing recognition has been given to the health-promoting potentials of oats and oat-derived bioactive compounds in recent years (Martínez-Villaluenga and Peñas, 2017).

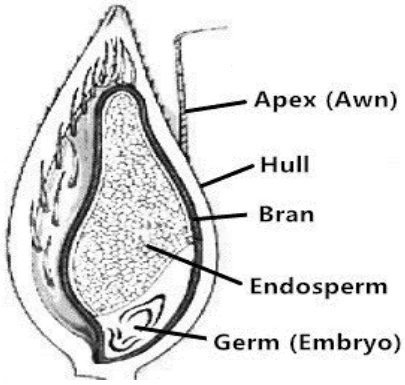


Figure 2.4 Cross section of an oat grain (Adapted from Butt *et al.*, 2008)

2.1.2 Nutritional compositions

Oat has a well-balanced nutritional profile. It is a good source of dietary fiber, unsaturated fatty acids and phytochemicals (Head *et al.* 2010). Nutritional components of oats and their reported concentrations are shown in Table 2.1. Oat starch comprises approximately 60% of oat grain. Notably, it has been reported that oats contain ~ 22% of slowly digestible starch and ~ 25% resistant starch of their total starch, which are important for maintaining blood glucose levels and improving intestinal health (Ovando-Martinez *et al.* 2013). Oat is also a good source of lipids, most of which are in the endosperm. Oat has higher lipid content, especially unsaturated fatty acids, than other cereals, which gives them advantages for being used as a high energy resource with beneficial fatty acid composition (Butt *et al.*, 2008). Moreover, phytochemicals in oats also play a significant role in health beneficial effects. Most of the phytochemicals have been found in oat bran, endosperm and germ (Rasane *et al.*, 2013). Antioxidants such as tocopherols are mostly found in oat germ and tocotrienols are mainly found in the endosperm, whereas most phenolic compounds are found in oat bran (Rasane *et al.*, 2013).

Table 2.1 Nutritional compositions of oat groats

Component	**Concentration**	**References**
Starch	60%	Berski *et al.* (2011)
Protein	11-15%	Robert *et al.* (1985)
Lipid	6-9%	Flander *et al.* (2007)
Total dietary fiber	10%	Butt *et al.* (2008)
	β-glucan: 2.3-8.5%	Flander *et al.* (2007)
Phytochemicals	Total tocols: 19.0-30.3 mg/kg	Peterson (2001)
	Phenolic acids: 469-472 mg/kg	Peterson (2001)
		Matilla *et al.* (2005)
	Flavonoids: trace amount	Matilla *et al.* (2005)

2.1.3 Oat antioxidants

Until recently oat grains have been recognized as important contributors of dietary antioxidants, even though they constitute a staple dietary component for most of the world's population (Lorenz and Lee, 1977). Although most of the health benefits of oats are attributed to the cholesterol-lowering potential of β-glucan, several phytochemicals have also been found and recognized for their positive impacts on human health, which include antioxidants and phenolic compounds (Peterson, 2001; Rasane *et al.*, 2015; Martínez-Villaluenga and Peñas, 2017). Because most phenolics are found in the bran layer of oat grains, oats could be a significant source of dietary antioxidants as they are normally consumed as whole-grain cereal (Peterson, 2001). Several phytochemicals have been found in oat groats that show antioxidant activity. The most abundant antioxidants are tocols (tocopherols and tocotrienols), phytic acids, phenolic acids, and avenanthramides (Avns) (Collins *et al.*, 1991; Peterson, 2001). Also, trace amounts of flavonoids and sterols are present in oat groats (Knights and Laurie, 1967; Andlauer & Furst, 1998).

Tocols are a group of lipid-soluble monophenols that are strong antioxidants generally called vitamin E, which are composed of a polar chromanol ring linked to an isoprenoid-derived hydrocarbon chain (Figure 2.2) (Tappel, 1962; Shahidi and de Camargo, 2016). A study found that total tocols concentrations ranged from 19.0-30.3 mg/kg among 12 oat genotypes in the U.S.A (Peterson and Qureshi, 1993), while another study reported a range of 16-94 mg/kg tocol found in European oat cultivars, with these differences in concentration being attributed to genotype and location (Redaelli *et al.*, 2016). The primary tocol of oat is α-tocotrienol with a lesser amount of α-tocopherol. Almost all of the tocopherols are found in the germ of oat groats, whereas most of the tocotrienols are located within the endosperm (Peterson, 1995).

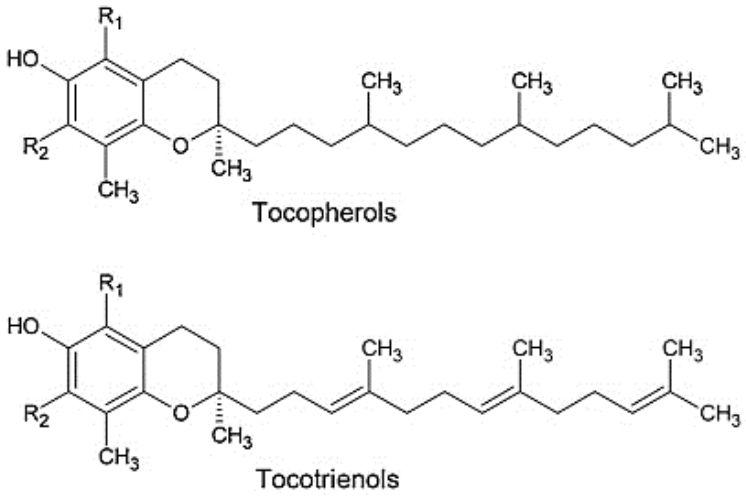


Figure 2.5 General structures of tocopherols and tocotrienols

Phenolic acids, which are also abundant in oats, are a group of hydroxyl derivatives of aromatic carboxylic acids. The concentration of total free phenolic acids in oat groats was found to be 8.7 mg/kg, which was much lower than soluble phenolic acid esters (20.6 mg/kg) and insoluble phenolic acids (57.7 mg/kg) (Sosulski *et al.*, 1982). Similarly, several studies identified traces of free ferulic, vanillic, and *p*-coumaric acids with less than 3 mg/kg in oats (Durkee and Thivierge, 1977; Dimberg *et al.*, 1996; Emmons and Peterson, 1999), whereas the bulk of ferulic, vanillic, sinapic, *p*-coumaric, and *p*-hydroxybenzoic acids were found as soluble or insoluble bound forms, which were released by alkaline hydrolysis or by β-glucosidase (Sosulski *et al.*, 1982). Thus, most phenolic acids in oat are present as simple soluble esters and complex insoluble esters conjugated with polysaccharides, proteins, or cell walls (Peterson, 2001). Phytic acid is another major antioxidant found in whole oat grains. It is a saturated cyclic acid that is the principle storage form of phosphorus in many plant tissues, especially bran and seeds (Lolas *et al.*, 1976). Although phytic acid is often considered as an anti-nutrient because it binds minerals in the digestive tract, making them less available to our bodies, it may also serve as a potent antioxidant because it suppresses iron-catalyzed oxidative reactions by forming an iron chelate (Saastamoinen *et al.*, 1992). Several studies have measured phytic acid concentrations in oat cultivars and found a range of 5.6-12.7 mg/g in oat groats (Lolas *et al.*, 1976; Miller *et al.*, 1980; Saastamoinen *et al.*, 1992). Another important group of antioxidants in oat are the avenanthramides. They were first identified in 1989 by Collins, who named them avenanthramides (Collins, 1989). They are a group

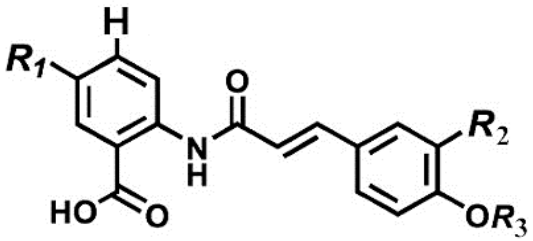
of phenolic compounds exclusively found in oats and exhibit high antioxidant activity. They will be discussed in detail in the section 2.1.4.

2.1.4 Avenanthramides: polyphenols found exclusively in oats

Avns are a group of low molecular weight, soluble phenolic compounds found exclusively in oats (Collins, 1989). Avns are conjugates of a phenylpropanoid (*p*-coumaric, ferulic, or caffeic acid) with anthranilic acid or 5-hydroxy anthranilic acid (Collins, 1989). The majority of Avns can be found in oat groats, with the highest concentration in the bran. More than 20 different forms of Avns have been identified in oats (Ishihara *et al.*, 1983; Collins, 1989; Bratt *et al.*, 2003; Peterson and Dimberg, 2008). The complete structures of the three most abundant avenanthramides have been determined and identified as Avn A, B and C (Collins, 1989; Figure 2.3). Chu *et al.* investigated concentrations of the three major Avns in seven common oat cultivars in Canada and found that the Avn A content ranged from 1.05-43.77 mg/kg, Avn B ranged from 3.66-58.63 mg/kg and Avn C ranged from 3.7-48.41 mg/kg (2013). Dimberg *et al.* (1996) reported the concentration ranges of Avn A, B and C in three Swedish oat cultivars as 25-47 mg/kg, 21-43 mg/kg and 21-43 mg/kg, respectively. In addition, four oat cultivars in U.S.A were also investigated for their Avn A, B and C contents and the average concentrations were found as 54 mg/kg, 36 mg/kg and 52 mg/kg, respectively (Emmons and Peterson, 1999).

A number of studies have been focused on investigating health promoting effect of Avns or Avn-enriched oat extracts. It has been found that Avns had strong antioxidant activities *in vitro* with Avn C being the highest (Dimberg *et al.*, 1993; Peterson *et al.*, 2002; Lee-Manion *et al.*, 2009; Yang *et al.*, 2014). Moreover, both oat phenolic-rich extracts and pure Avns showed anti-inflammatory activity by reducing the expression of pro-inflammatory cytokines and repressing NF-κB function (Guo *et al.*, 2008; Sur *et al.*, 2008; Chu *et al.*, 2013; Yang *et al.*, 2014). In addition, Avn C was reported to inhibit the proliferation of rat vascular smooth muscle cells through modulation of cell cycle, indicating the potential health benefit of oat consumption in the prevention of coronary heart disease (Nie et al, 2006a; 2006b). Notably, the structures of Avns are very similar to a synthetic anti-allergic drug called Tranilast (Figure 2.3), which also has been found to have anti-proliferative effect on rat vascular smooth muscle cells (Tanaka *et al.*, 1994). Recently, the antiproliferative effects of Avns have also been examined on several cancerous cell

lines and found that Avn C was able to decrease the proliferative rates of colonic cancer cell lines, including Caco-2, HT29, LS174T, HCT116 (Guo *et al.*, 2010) and MDA-MB-231 breast cancer (Hastings and Kenealey, 2017). These findings make Avns promising candidates for therapeutic use and the development of nutraceutical products.



Compound	R_1	R_2	R_3
Avn A	OH	H	H
Avn B	OH	OCH_3	H
Avn C	OH	OH	H
Tranilast™	H	OCH3	CH3

Figure 2.6 Chemical structures of Avn A, B & C and Tranilast (Adapted from Peterson, 2001)

2.2 Oxidative stress and antioxidant defense systems

2.2.1 Free radicals, oxidative stress and human diseases

A free radical is any atom or molecule that has a single unpaired electron in an atomic orbital (Bagchi and Puri, 1998). Free radicals and other reactive oxygen species (ROS) are derived either from normal metabolic processes in the human body or from external sources such as X-rays, ozone, cigarette smoking, air pollutants, and industrial chemicals (Bagchi and Puri, 1998; Lobo *et al.*, 2010). Many ROS are unstable and highly reactive, such as hydroxyl radicals ($^{\bullet}OH$), superoxide anions ($O_2^{\bullet -}$) and H_2O_2. They cause cellular damage by attacking important macromolecules leading to homeostatic disruption. Targets of free radicals in the body include lipids, nucleic acids, proteins and cell membranes (Yong and Woodside, 2001). Several antioxidant defense systems (detailed discussion in 2.2.2 and 2.2.3) are responsible for regulating ROS and maintaining the survival and health of cells or organisms; however, when the production of ROS exceeds the capacity of the antioxidant defense, oxidative stress occurs, leading to the a number of human diseases (Bagchi and Puri, 1998; Yong and Woodside, 2001). It has been found and verified by numerous scientific evidence that free radicals are strongly associated with more than sixty different health conditions, including the aging process (Finkel and Holbrook, 2000), cancer (Wiseman and Halliwell, 1996; Kennedy *et al.*, 1998), diabetes (Leinonen *et al.*, 1997;

Hannon *et al.*, 1998), Alzheimer's disease (Price *et al.*, 1998; Huang *et al.*, 2016), stroke (Allen and Bayraktutan, 2009), heart attack and atherosclerosis (Napoli, 1997; Fu *et al.*, 1998). As such, antioxidant defenses are absolutely critical for disease prevention and human health.

2.2.2 Antioxidant defense systems

2.2.2.1 Enzymatic antioxidants

Several endogenous defense mechanisms have been identified to help protect against free radical-induced cell damage in our body. Antioxidant enzymes are one of the endogenous components that stabilize or deactivate free radicals before they cause cellular damage (Matés *et al.*, 1999). They stabilize free radicals by reducing the energy of free radicals or by donating electrons for pairing. In addition, they may also interrupt the oxidizing chain reaction to minimize the damage caused by free radicals (Matés *et al.*, 1999). Enzymatic antioxidants include superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) and glutathione synthetase (GSS), and their biosynthesis can be induced by H_2O_2 inside cells (Krishnamurthy and Wadhwani, 2012).

SOD is a group of metal-containing antioxidant enzymes that can add or remove an electron from highly reactive $O_2^{\bullet -}$ to generate the less reactive species oxygen (O_2) and H_2O_2, with the latter further degraded by other antioxidant enzymes (Matés *et al.*, 1999; Figure 2.4). Three isoforms of SOD were found in all eukaryotic cells and have been identified as SOD1 (cytosolic Cu/Zn-SOD), SOD2 (mitochondrial Mn-SOD) and SOD3 (extracellular SOD) (Matés *et al.*, 1999). SOD2 (Mn-SOD) is a homotetramer found in mitochondria that contains one manganese ion per subunit. As the mitochondrion is a major site for cellular respiration using oxygen, SOD2 plays an essential role for clearing mitochondrial oxygen radicals and, as a result, protect against cell damage or death. SOD1 (Cu/Zn-SOD) is a homodimer with a Cu/Zn site in each subunit (Krishnamurthy and Wadhwani, 2012). It is believed to play a major role in the first line of antioxidant defense. Notably, SOD1 may not be essential for life as Cu/Zn-SOD knockout mice appeared normal and exhibited differences only after traumatic injury, whereas Mn-SOD knockouts died in three weeks (Li *et al.*, 1995). SOD3 is an extracellular SOD that can be found in extracellular fluids such as plasma and lymph. SOD3 is a Cu/Zn containing antioxidant enzyme,

but unlike SOD1, it is regulated by cytokines instead of oxidants (Krishnamurthy and Wadhwani, 2012).

Peroxide generated by SODs can be subsequently converted to water by CAT or GPx catalytic reactions (Matés *et al.*, 1999). CAT is a tetrameric enzyme that contains a ferriprotoporphyrin per subunit (Putnam *et al.*, 2000). It efficiently reacts with H_2O_2 to form water and oxygen as shown in Figure 2.4. GPx is a selenium-containing antioxidant enzyme that uses glutathione (GSH) to catalyze the reduction of peroxide (Otto *et al.*, 1983; Matés *et al.*, 1999; Figure 2.5), thereby protecting cells from damage caused by oxidative stress. As a result, SODs, GPx and CAT work together to remove superoxide and peroxides before they cause cellular damage.

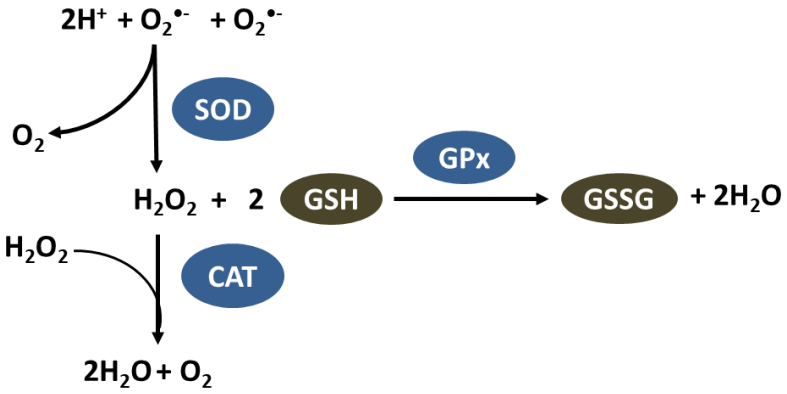


Figure 2.7 Enzymatic reactions catalyzed by superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Adapted from Borowicz *et al.*, 2016)

2.2.2.2 Non-enzymatic antioxidants

Non-enzymatic antioxidant defense systems can be classified into two categories: metabolic antioxidants and dietary antioxidants. Metabolic antioxidants are non-enzymatic molecules inside cells or blood plasma that provide rapid inactivation of free radicals and oxidants, including but not limited to, glutathione, myoglobin, ferritin, uric acid, and albumin (Mirończuk-Chodakowska *et al.*, 2018). Dietary antioxidants are exogenous antioxidants such as ascorbic acid, vitamin A, vitamin C, α-tocopherol, and plant polyphenols, which are usually obtained from consumed fruits, vegetables, beverages and cereal products (Mirończuk-Chodakowska *et al.*, 2018).

Usually endogenous antioxidant defense systems are inadequate without engaging exogenous reducing compounds such as vitamin C, vitamin E, carotenoids and polyphenols. Polyphenols are the most abundant antioxidants in our diet and they are well recognized for their antioxidant properties. Phenolic rings are the functional groups capable of accepting free radicals, becoming phenoxyl radicals (Flora, 2009). These radicals are stabilized by resonance delocalization of the electrons within the aromatic ring and create quinones. In addition, the phenoxyl radicals can also enter into other reactions where the radicals could be neutralized, such as dimerization or radical substitution (Flora, 2009). In this way, subsequent free-radical chain reactions are inhibited, and oxidation is retarded. A general mechanism is shown in Figure 2.5.

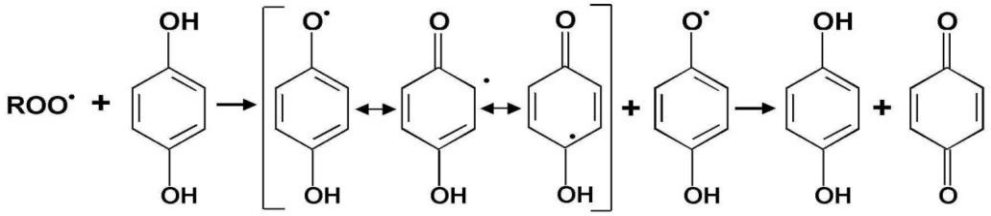


Figure 2.8 Phenolic antioxidant reaction mechanism (Adapted from Kurek-Gorecka *et al.*, 2014)

2.2.3 Plants as sources of dietary antioxidants

Plants develop their antioxidant systems in an evolutionary manner based on their need to survive oxidative stresses generated from the environment (e.g. temperature and light) and themselves (transition metals) (Nakatani, 2003). A wide variety of naturally occurring antioxidants in plants have been identified with differences noted in structure, physical and chemical properties, mechanism of action and site of activity (Brewer, 2011). Polyphenols are the most abundant antioxidants in our diet. Their total dietary intake could be as high as 1 g/d, which is much higher than all other types of dietary antioxidants. For example, the daily intake of polyphenols in our diet is ~10 times higher than the intake of vitamin C and 100 times higher than the intakes of vitamin E and carotenoids (Manach et al, 2004). In our diet, the main sources of polyphenols are fruits, vegetables, whole grains and other types of foods and beverages derived from them, such as red wine (rich in resveratrol), extra virgin olive oil (rich in hydroxytyrosol), chocolate and tea, particularly green tea (rich in epigallocatechin gallate) (Scalbert and Williamson, 2000). Most polyphenols derived from plants possess excellent antioxidant properties and several polyphenols can also function as signaling molecules that regulate gene expression (Gillespie *et al.*, 2016).

2.2.4 Determination of antioxidant activities

2.2.4.1 DPPH & ABTS free radical scavenging activities

Antioxidant activity (capacity or potential) is widely used as a parameter to characterize different plant foods (fruits, vegetables, wines, teas, oils, etc.). This activity is based on the chemical composition of the foods materials that are capable of protecting a biological system against the potentially harmful effects of processes and reactions such as oxidation and the production of reactive oxygen and nitrogen species (Huang *et al.* 2005). *In vitro* free radical scavenging tests are one of the most common methods used to measure the antioxidant activity of a biological material/extract. The two most widely used chemical compounds to measure the antioxidant activity of biological materials are the chromogens, $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radicals. The DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate; Figure 2.6-a) free radical method is an antioxidant assay based on an electron-transfer mechanism that produces a violet solution in a protic solvent such as ethanol (Huang *et al.* 2005). The DPPH radical is stable at room temperature and is reduced in the presence of an antioxidant, leading to a decrease in the intensity of the initial colour of the sample solution. The ABTS free radical assay is based on the scavenging ability of antioxidants for the stable radical cation $ABTS^{\bullet+}$ [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); Figure 2.6-b]. The mechanism of the reaction is based on electron transfer from sample antioxidants to $ABTS^{\bullet+}$ and it measures the ability of sample antioxidants that react directly with the $ABTS^{\bullet+}$ radical to decrease the reaction solution colour (Sharma and Singh, 2013).

a. b.

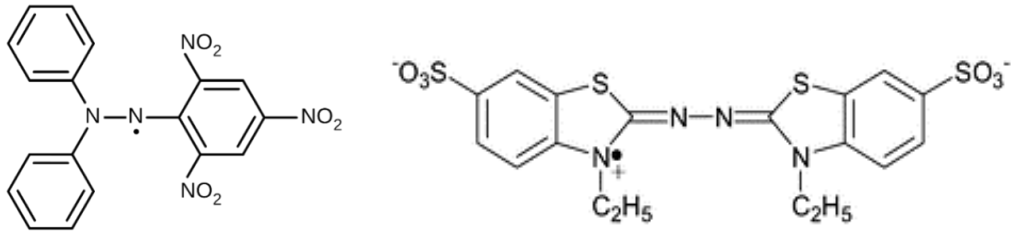


Figure 2.9 Chemical structures of $DPPH^{\cdot}$ free radical (a) and $ABTS^{\bullet+}$ free radical (b) (Adapted from Huang *et al.* 2005)

2.2.4.2 Intracellular antioxidant activity

Although DPPH and ABTS assays provide a relatively simple way to estimate the antioxidant activities of compounds, they do not mimic biological systems. Without data on their bioavailability and cellular impact, the relevance of this information to antioxidant effectiveness in organisms is very limited. Therefore, an understanding of how antioxidants perform within cells is essential. Exogenous antioxidants are mainly derived from food sources such as vitamins and polyphenols, and they have to be absorbed and distributed to the sites where the radicals are, in order to be effective (Matés *et al.*, 1999). Several polyphenols have been proven to have the ability to effectively scavenge free radicals before they cause damage to cell components.

One of the fastest and accessible methods for monitoring ROS production intracellularly is based on the detection of ROS-sensitive fluorescent probes. These probes react with ROS, forming intermediate probe-derived radicals that are successively oxidized to generate the corresponding fluorescent products (Wardman, 2007). In addition, intracellular antioxidant activity can also be evaluated through antioxidant enzyme activities. Several studies have shown polyphenols, such as quercetin and resveratrol, have the ability to enhance the activity of antioxidant enzymes inside cells so as to increase the intracellular antioxidant activity (Jang and Surh, 2001). Chen and colleagues reported that an avenanthramides-enriched mixture extracted from oat increased human plasma GSH expression by ~14% (Chen *et al.*, 2007).

2.3 Inflammation

Inflammation is an essential part of the body's immune systerm responsible for removal of harmful stimuli and initiating the healing process. It is a major defense mechanism in the process to eliminate endogenous or foreign stimuli, such as a virus, bacteria and damaged cells, and initiate tissue repair (Medzhitov, 2008). There are two types of inflammation in humans: acute and chronic inflammation. Acute inflammation arises rapidly in response to harmful stimuli and is only present for a short term from hours to days. Without acute inflammation, wound healing would be impaired, and infections could become lethal; however, if inflammation persists longer than necessary, it can bring more harm than benefit (Medzhitov, 2008). Prolonged inflammation, known as chronic inflammation, refers to low-grade long-term inflammation and can last for several months and even years.

2.3.1 Chronic inflammation

Chronic inflammation can be caused by a number of factors. If the acute inflammatory response fails to eliminate the harmful stimuli, the inflammatory process persists and turns into chronic inflammation (Kim *et al.*, 2006). Chronic inflammation can also result from autoimmune responses that mistake own antigens as pathogens, leading to the attack of normal healthy tissues. Notably, chronic inflammation is widely observed in obesity. Studies revealed that fatty deposits can trigger autoimmune response, in which the immune system mistakes fatty deposits for intruders and starts to attack adipose cells (Greenberg and Obin, 2006; Rogowski, 2010). The damaged adipose cells recruit macrophages and assist in releasing inflammatory cytokines, including Tumor necrosis factor α (TNFα) and interleukin 6 (IL-6), which further induce inflammation (Greenberg and Obin, 2006; Stolarczyk, 2017). Moreover, chronic inflammation is often associated with environmental or habitual factors, such as excess weight, poor diet, lack of exercise, stress, smoking, industrial chemicals and pollution (Ruiz-Núñez *et al.*, 2013). Unlike acute inflammation, chronic low-grade inflammation often does not have symptoms, but it can eventually cause many diseases and conditions such as diabetes, rheumatoid arthritis, atherosclerosis and cancer (Salminen *et al.*, 2008).

Increasing evidence has shown that diabetes is an inflammatory disease (Zhong *et al.*, 2017). Type 1 diabetes is caused by autoimmune-mediated chronic inflammation, leading to the destruction of pancreatic β cells and insufficient secretion of insulin (Batch, 1994). Type 2 diabetes, characterized by insulin resistance, has been linked to obesity and chronic low-grade inflammation in peripheral tissues (e.g. skin) (Hotamisligil *et al.*, 1993). Chronic inflammation is also strongly associated with cardiovascular diseases. When cholesterol deposits in the lining of blood vessels, it is recognized as an insult and triggers inflammatory responses. Fatty plaques accumulate in inflamed blood vessels and cause blockages and blood clots, which leads to heart attacks (Libby, 2006). In addition to diabetes and cardiovascular diseases, bone loss is another consequence of chronic inflammation. It has been shown that cytokines produced by chronic inflammation impair bone remodeling, in which damaged parts of bones are replaced with new ones (Hardy and Cooper, 2009). Furthermore, intestinal inflammation can interfere with calcium and vitamin D absorption, which are important to bone health (Irwin *et al.*, 2016).

2.3.2 Inflammatory cytokines

Inflammation is controlled by a number of extracellular signaling mediators and regulators, including cytokines, growth factors and peptides. Cytokines are a group of small proteins released by cells that act as chemical messengers for communications and interactions between cells. They are key modulators of inflammation, participating in both acute and chronic inflammation through a complex network of interactions and signaling pathways (Turner *et al.*, 2014). Cytokines are produced by a broad range of cells, including immune cells like macrophages, B lymphocytes, T lymphocytes, as well as endothelial cells, fibroblasts, and various stromal cells (Lawrence, 2009). Cytokines are commonly classified as pro-inflammatory cytokines and anti-inflammatory cytokines. Pro-inflammatory cytokines such as interleukin 1 beta (IL-1β), IL-6, interleukin 8 (IL-8) and TNFα promote inflammatory responses, whereas anti-inflammatory cytokines such as IL-1 receptor antagonist, and interleukins 4, 10, 11 and 13 are responsible for supressing the activity and synthesis of pro-inflammatory cytokines (Turner *et al.*, 2014). A balance between pro-inflammatory and anti-inflammatory cytokines is important to maintain cellular and organismal health.

2.3.3 NF-κB signaling

NF-κB is a family of eukaryotic nuclear transcription factors that regulate the transcription of deoxyribonucleic acid (DNA) and are involved in activation of the genes related to inflammatory and immune responses (Li and Verma, 2002). There are two pathways for the activation of NF-κB, known as the classical (canonical) pathway and the alternative (non-canonical) pathway. NF-κB consists of p65 and p50 subunits, and it is mainly involved in natural immunity and general inflammation (Li and Verma, 2002). Inhibitor of κB (IκB) is an protein that is bound to NF-κB and inhibits its function under non-stress conditions. Upon stress, IκB is phosphorylated and degraded by 26S proteasome, liberating the active NF-κB transcription factor subunits to translocate to the nucleus and drive the expression of genes encoding pro-inflammatory cytokines, such as IL-1β, IL-6, IL-8 and TNFα (Gilmore, 2006; Figure 2.7). Activation of NF-κB leads to the inflammation process involved in many diseases, such as asthma, rheumatoid arthritis and bowel disease (Yamamoto and Gaynor, 2001). Plant polyphenols such as curcumin, resveratrol

and green tea catechins have been found to be potent inhibitors of NF-κB by preventing the degradation of IκB (Aggarwal and Shishodia, 2006).

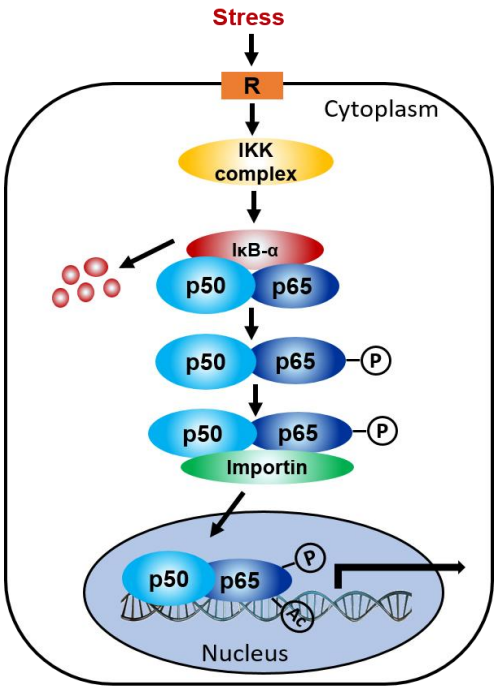


Figure 2.7 Canonical NF-κB activation pathways. IKK: IκB kinase; R: receptor (Adapted from Lawrence, 2009)

2.4 Nrf2/HO-1 system in cytoprotection

Heme oxygenase (HO), encoded by the *HMOX* gene, is an evolutionarily conserved enzyme that catabolizes heme into ferrous (Fe^{2+}), carbon monoxide (CO), and biliverdin (Tenhunen *et al.*, 1968; Figure 2.8). Two isoforms of HO have been identified; one is the constitutively expressed form (HO-2) and the other is the inducible form (HO-1). Cumulative evidence has shown that HO-1 is one of the most critical cytoprotective enzymes in human (Morse and Choi, 2005; Gozzelino *et al.*, 2010). The major biological function of HO is the degradation of free heme. Under oxidative stress, heme groups can be released from hemoproteins and catalyze the production of free radicals, leading to cellular damage, and HO-1 keeps free heme at a relatively low level for cellular protection (Gozzelino *et al.*, 2010). Besides the reduction of free heme, HO-1 also exerts cytoprotective effects through the generation of three metabolites during the heme degradation process that are involved in health promotion (Figure 2.8). Ferrous ions produced by HO activity upregulates the expression of ferritin, which catalyzes the oxidation of

iron from the ferrous form (Fe^{2+}) to the ferric form (Fe^{3+}) (Harrison and Arosio, 1996). In addition, ferritin can bind free heme that prevents cellular damage (Harrison and Arosio, 1996). Carbon monoxide generated from heme degradation has shown anti-inflammatory, antiapoptotic, and antiproliferative effects through regulating p38β and mitogen-activated protein kinase (MAPK) pathways (Piantadosi, 2008). Additionally, CO protective effects were observed in many cell types through decreased levels of ROS (Piantadosi, 2008). Biliverdin, the precursor of bilirubin, has been reported to have antiviral activity, and bilirubin is now recognized as a potent antioxidant in the body (Stocker *et al.*, 1987). A number of studies observed that *HMOX 1* knockout mice developed severe pathologic conditions, resulting in a high incidence of mortality (Poss and Tonegawa, 1997; Wiesel *et al.*, 2000), indicating that HO-1 plays an important role on maintaining homeostasis of normal physiological conditions (Soares and Bach, 2009)

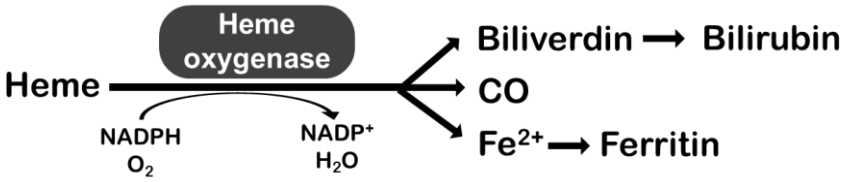


Figure 2.10 Heme degradation catalyzed by heme oxygenase (Adapted from Soare and Bach, 2009)

NF-erythroid 2-related factor 2 (Nrf2) is a transcription factor that regulates expression of many antioxidant and detoxifying enzymes including HO-1 (Itoh *et al.*, 1997). Under unstimulated conditions, Nrf2 is sequestered by its inhibitor Kelch-like-ECH-associated protein 1 (Keap1) in cytoplasm that inhibits translocation of Nrf2 to the nucleus, and Nrf2 is subject to proteasomal degradation by ubiquitin (Ub) (Itoh *et al.*, 1997; Niture *et al.*, 2009). Nrf2 becomes transcriptionally active through multiple mechanisms including direct phosphorylation and loss of contact between Nrf2 and Keap1. Upon activation, Nrf2 is able to enter the nucleus and bind antioxidant response element (ARE) together with Maf protein to drive the expression of target genes such as *HMOX1* and *NQO1* (Itoh *et al.*, 1997; Ma, 2013). As a result, Nrf2 activation leads to decreased overall oxidative stress and inflammation. A widely accepted model for induction of ARE-mediated antioxidant gene expression involves phosphorylation of serine/threonine residues of Nrf2 by protein kinases, leading to enhanced nuclear accumulation of Nrf2 and subsequent ARE

binding (Niture *et al.*, 2009). A general mechanism of Keap1-Nrf2 system under stressed and unstressed conditions is shown in Figure 2.9.

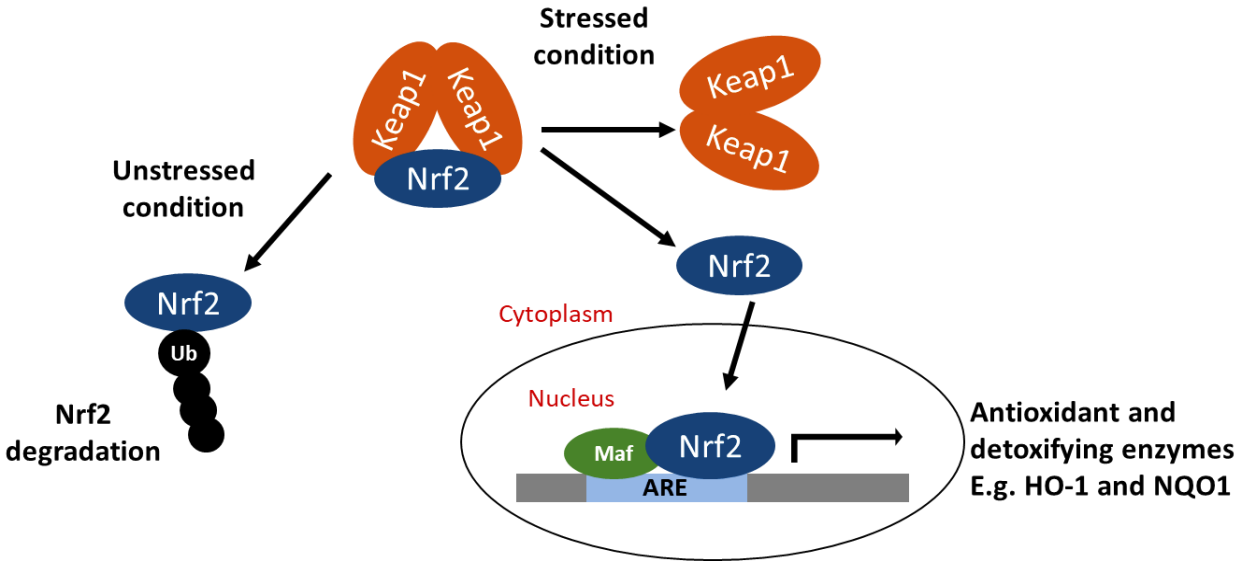


Figure 2.11 Keap1-Nrf2 system for the regulation of cytoprotective genes. (Adapted from Ma, 2013)

2.5 Nutrient sensing at cellular level

Nutrition can positively influence human health and lower the risks associated with exposure to mixtures of environmental chemicals. In nutrigenomics, nutrients are seen as signaling molecules that function in a specific cell in the body (Neeha and Kinth, 2013). The nutrients are detected by many sensor systems in cells. The sensory systems that interpret information from nutrients include transcription factors. Once the nutrient interacts with such a sensory system, it impacts gene expression and metabolite production. As a result, different nutrients change different patterns of gene expression and metabolite production (Van Ommen and Stierum, 2002). Current research is now focused on how nutrients impact the expression and action of specific genes and how these proteins in turn affect the response to nutrients. Although numerous bioactive compounds have been shown to have health-promoting potential, more scientific research needs to be conducted before we can begin to make science-based dietary recommendations. This scientific evidence will provide consumers with food choices for disease prevention and improved health that match their lifestyles, cultures and genetics.

2.5.1 Polyphenols on gene regulation

Phenolic compounds in foods have attracted great interest recently due to growing evidence of their beneficial effects on human health. Polyphenols, which are the most abundant antioxidants in our diet, possess a wide variety of structures, and physical and chemical properties (Brewer, 2011). Most of the evidence on the beneficial effects of dietary polyphenols is derived from experiments performed *in vitro* or in animal models. Many polyphenols have been demonstrated to have anti-inflammatory, anti-allergenic, anti-aging, and anticarcinogenic activities (Guo *et al.*, 2009). The broad therapeutic potentials of most polyphenols are largely attributed to their antioxidant properties. In addition to an antioxidant effect, several polyphenols may act as signaling molecules that influence gene expression independent of their free radical scavenging activity (Aggarwal and Shishodia, 2006; Gillespie *et al.*, 2016).

Resveratrol (3,4',5-trihydroxy-*trans*-stilbene) consists of two aromatic rings which are attached by a methylene bridge. It is one of the most well-known and well-studied polyphenols commonly found in red wine, red grape skin, peanuts and berries (De la Lastra and Villegas, 2005). Increasing research interest on resveratrol started from the "French paradox", which describes improved cardiovascular health in French people despite their high-fat diets. Since then, resveratrol has been explored for its health-promoting and disease prevention effects. Numerous studies show that resveratrol exhibits antioxidant, anti-inflammatory, antiproliferative and anti-aging potentials from both *in vitro* and *in vivo* experiments (Zhu *et al.*, 2011; Minagawa *et al.*, 2015; Park *et al.*, 2016). Besides the free radical scavenging capability of resveratrol, an important antioxidant mechanism of resveratrol has been reported as the suppression of the pro-oxidative enzyme NADPH oxidase and the induction of antioxidant enzymes SOD1 and GPx1 (Spanier *et al.*, 2009). Moreover, resveratrol also supresses inflammation through reducing NF-κB activation or inhibiting translocation of NF-κB from the cytosol to the nucleus (Zhu *et al.*, 2011; Minagawa *et al.*, 2015). This inhibitory effect is likely due to the activation of Sirtuin 1 (SIRT1)-mediated deacetylation of p65 subunit of NF-κB, phosphorylation of NF-κB and stabilization of IκB (inhibitor of NF-κB), leading to the downregulation of pro-inflammatory cytokine gene expression (De la Lastra and Villegas; 2005; Zhu *et al.*, 2011). Recent research has been driven to exploration of anti-aging potential of resveratrol. In 2003, Howitz and Sinclair showed that resveratrol significantly extends the lifespan of *Saccharomyces cerevisiae* (Howitz *et al.*, 2003). Later the positive effect of resveratrol on the lifespan has been observed in a worm *Caenorhabditis elegans,*

a fruit fly *Drosophila melanogaster* (Wood *et al.*, 2004; Gruber *et al.* 2007) and a short-lived fish *Nothobranchius furzeri* (Valenzano *et al.*, 2006). The mechanisms for the lifespan extension are associated with the activation of Sirtuin deacetylases directly or indirectly by resveratrol (Pacholec *et al.*, 2010). The activation of Sirtuin by resveratrol also associates with activation of autophagy, which is mediated by SIRT1 activation and the mammalian target of rapamycin (mTOR) inhibition (Park *et al.*, 2016).

Epigallocatechin gallate (EGCG), also known as epigallocatechin-3-gallate, is another well-known dietary polyphenol mostly found in green tea. It is a type of catechin and made of the ester of epigallocatechin and gallic acid. Various health benefits and molecular targets of EGCG have been proposed based on studies in cell lines and animal models. Similar to resveratrol, EGCG can reduce cellular oxidative stress by up-regulating antioxidant enzymes through Nrf2/ARE pathway (Zheng *et al.*, 2012). In addition, EGCG decreases inflammatory response by inhibition of NF-κB pathway and regulation of cyclooxygenase 2 (COX-2), thereby reducing the expression of genes encoding pro-inflammatory cytokines (Li *et al.*, 2004). EGCG has also been reported to function as a signaling molecule that triggers cascades of metabolic pathways, leading to the inhibition of carcinogenesis. The proposed mechanisms include the inhibition of MAPK and phosphoinositide 3-kinase/ protein kinase B (PI3K/AKT) pathways, NF-κB and AP-1-mediated transcription, growth factor-mediated signaling (Gupta *et al.*, 2003; Khan *et al.*, 2006; Nandakumar *et al.*, 2011). Also, EGCG impacts gene expression by epigenetic modification, which also leads to inhibition of tumor cell growth or induction of apoptosis (Fang *et al.*, 2003; Nandakumar *et al.*, 2011). Recently, Holczer and colleagues (Holczer *et al.*,2018) found that EGCG promotes autophagy-dependent survival through regulating mTOR/5' AMP-activated protein kinase (AMPK) pathways.

Most of the evidence on the health-promoting effects of dietary polyphenols is derived from experiments using concentrations much higher than those generally attainable through diet use. Moreover, the polyphenol compounds used in those experiments are often aglycones or their sugar conjugates, rather than their effective metabolites. Therefore, more evidence is needed to show the effectiveness of polyphenols in disease prevention and human health including their bioavailability and the fate of their metabolites and evaluate their biological activity in different target tissues.

3. MATERIALS AND METHODS

3.1 Oat samples

Oat samples of ten varieties and breeding lines were obtained from the Crop Development Centre, University of Saskatchewan. They were BW10, BW513, BW5303, CDC Dancer, CDC Morrison, CDC Sol-Fi, Jumbo, Marion, Newberg, OT2021.

3.2 Exaction of phenolic compounds from oat samples

Five (± 0.01) grams of ground powder of each sample was extracted three times with 50 mL of methanol:phosphoric acid solvent mixture (80:20, v:v; pH 2.1) by magnetic stirring for 30 min. The sample mixtures were centrifuged for 10 min at 10,000 rpm and the supernatants were pooled and evaporated at 40 °C using a Buchi III Rotavapor vacuum evaporator (Brinkmann Instruments, Mississauga, ON). The residue was re-suspended in 5 mL of the extraction solvent and centrifuged at 10,000 rpm for 10 min. The final volume of the oat extract was made up to 5 mL in a volumetric flask with methanol. The oat extract samples were stored in dark at -18 °C for future analysis.

3.3 HPLC analysis of Avn A, B and C in oat extracts

Reversed phase HPLC was performed on an 1100 series HPLC system (Agilent Technologies Canada Incorporated, Mississauga, ON), equipped with a photodiode array (PDA) detector. Phenolic separation was achieved using a 250 x 4.6 mm Luna® 5 μm, C18 column (Phenomenex, Torrance, CA, USA) in series with a C18 guard cartridge (Phenomenex, Torrance, CA, USA). Mobile phases consisted of solvent A: H_2O with 5% acetonitrile and 0.5% trifluoracetic acid by volume; Solvent B: acetonitrile. Samples were run with a linear gradient over 40 min from 5 to 40 % solvent B in solvent A with a flow rate of 1 mL/min followed by 40 % solvent B in solvent A for 5 min. The injection volume was 10 μL. All samples were syringe filtered (0.22 μm pore size; 13 mm diameter; Sigma-Aldrich, Oakville, ON, Cat #: F7148) prior to HPLC analysis. Standards run in conjunction with the samples included: Avn A (SynInnova, Edmonton, AB, Cat #: SL707), Avn B (SynInnova, Cat #: SL337) and Avn C (SynInnova, Cat #: SL340). Standards were prepared at the following concentrations: 2.0, 4.0, 6.0, 8.0, 10.0, 20.0 and 40.0 (± 0.02) ppm.

Detection was monitored at 340 nm with reference at 700 nm. All samples were analyzed in triplicate.

3.4 Total phenolic content of oat extracts

The Folin-Ciocalteau (FC) method was used to determine total phenolic content of oat extract samples. The method was adapted from Singleton *et al.* (1999). In brief, a 2.0 N stock FC solution (Sigma-Aldrich, Cat #: 47641) was diluted 1/10 with water obtained Millipore Milli-Q™ water system (Millipore Corporation, Milford, MA, USA). All oat extracts were diluted 1/20 with Milli-Q™ water. One hundred and fifty microliters of the diluted oat extract was mixed with 750 µL diluted FC reagent by vortexing. Six hundred microliters of 15% (w/v) Na_2CO_3 solution was then added followed by vortexing. The samples were then held static at room temperature in the dark for 2 h. Following the incubation period, samples were analyzed by UV-visible spectroscopy (Genesys 10S UV-visible, Fisher Scientific, Edmonton, AB) at 765 nm. A control was prepared which contained all the reagents and 150 µL Milli-Q™ water in place of the diluted samples. Milli-Q™ water was used as blank. A standard curve was prepared using gallic acid at concentrations ranging from 10-50 ± 0.2 mg/L in water, which were analyzed in conjunction with samples. Standard curves had correlation coefficients ≥0.9989. All samples and standards were analyzed in triplicate and the results were reported as gallic acid equivalent (GAE).

3.5 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activity

$ABTS^{\bullet+}$ free radical solution was prepared by mixing 7.0 mM ABTS solution (Sigma-Aldrich, Cat #: 11557) and 2.45 mM potassium persulfate (Sigma-Aldrich, Cat #: 216224) in a ratio of 1:1. This mixture was held static at room temperature for 12 h in the dark to afford $ABTS^{\bullet+}$ formation. The resulting $ABTS^{\bullet+}$ radical cation solution was diluted approximately 1 in 50 with 70% (v/v) methanol to give an absorbance reading of 0.75 ± 0.05 at 734 nm. Each oat extract sample was diluted 1/4, 1/3, 1/2, 1/1 with methanol. The Trolox standard (Sigma-Aldrich, Cat #: 238813) was prepared in 70% (v/v) methanol at the following concentration range: 0.1 (0.4 mM) to 0.5 mg/mL (2.0 mM). Avn A, B and C were prepared from 100 ppm to 1000 ppm in methanol. Caffeic acid (Sigma-Aldrich, Cat #: C0625), gallic acid (Fisher Scientific, Cat #: A122-500) and

Trolox (Sigma-Aldrich, Cat #: 238813) were used as standards and prepared from 50 to 300 ppm in methanol.

The assay was conducted by mixing 10 µL of the sample solution with 1.0 mL of $ABTS^{\bullet+}$ solution. The absorbance was read at 734 nm at time intervals of 1, 4 and 6 min. A control solution of 10 µL 70% (v/v) methanol in 1.0 mL $ABTS^{\bullet+}$ solution was prepared and analyzed at the same wavelength. Absorbance values measured at 6 min were used to calculate the percent radical inhibition as follows:

$$\% \text{ ABTS inhibition} = [1-(A_{734nm\ sample})/ (A_{734nm\ control})] \times 100$$

Where:

$A_{734nm\ sample}$ = sample absorbance at 734 nm

$A_{734nm\ control}$ = control absorbance at 734 nm

The % $ABTS^{\bullet+}$ inhibition was plotted as a function of sample concentration and resulting linear regression equations were determined. Correlation coefficients of the linear regression equations were ≥ 0.99. The 50% radical inhibition concentration (IC_{50}) was determined by the concentration that resulted in 50% radical scavenging activity using the linear regression curve. All samples were analyzed in in triplicate.

3.6 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

A 500 µM DPPH solution was prepared by dissolving 9.8 ± 0.2 mg of DPPH (Sigma-Aldrich, Cat #: D9132) in 70% (v/v) methanol. Fresh DPPH solution was prepared daily for sample analysis. Oat extracts were diluted 1/4, 1/3, 1/2 with methanol. Avn A, B and C were prepared from 100 to 1000 ppm in methanol. Caffeic acid, gallic acid and Trolox were used as references and prepared from 50 to 300 ppm in methanol.

A 1.0 mL DPPH solution and 0.25 mL sample solution were mixed by vortexing. The blank consisted of methanol, and the control was prepared by mixing 1.0 mL DPPH solution with 0.25 mL of methanol. All samples were held static at room temperature in the dark for 15 min, prior to absorbance measurement at 517 nm. The % DPPH radical scavenging activity was calculated as follows:

% DPPH radical scavenging activity= [1-($A_{517nm\ sample}$)/ ($A_{517nm\ control}$)] ×100

Where:

$A_{517nm\ sample}$ = sample absorbance at 517nm

$A_{517nm\ control}$ = control absorbance at 517nm

The IC_{50} was determined at the concentration that resulted in 50% radical scavenging activity using the linear regression curve of % DPPH vs sample concentration. All samples were analyzed in triplicate.

3.7 Cell culture

Normal human skin fibroblasts 2DD cells (previously described in Bridger *et al.* 1993) were grown in high glucose (4.5 mg/mL) Dulbecco's Modified Eagle Medium (DMEM) (pH 7.7; Corning, Manassas, VA, USA, Cat #: ca45000-304) supplemented with 10% (w:w) fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, Cat #: 12483-020) and 1.0% (w:w) penicillin streptomycin (GE Healthcare Life Sciences, Logan, UT, USA, Cat #: SV30010). Cells were seeded at an initial density of 3000 cells/cm^2 and incubated at 37°C in a humidified with 5% carbon dioxide (CO_2). Cells were passaged every 3 to 4 days with cells never excessing 80% confluency. Cells from passage number 12 to18 were used in experiments.

Four human cancer cell lines: MCF-7 cells (human breast adenocarcinoma), HCT 116 cells (human colon carcinoma), HepG2 cells (human liver hepatocellular carcinoma) and U2OS cells (human bone osteosarcoma) were purchased from American Type Culture Collection (Manassas, VA, USA) grown in the same media and under the same culture condition as 2DD cells. Culture media was changed every 3 to 4 days.

3.8 Cell treatment

Media used for Avn C treatments was identical to that used for proliferative cultures. Avn C stock solution was made by dissolving Avn C in dimethyl sulfoxide (DMSO) to a final concentration of 50 mM. DMSO was used as a vehicle control and never exceeded 0.4% (v:v) in culture media.

3.8.1 Cell treatment for intracellular antioxidant activity assays

2DD cells were seeded at a density of 3,000 cells/cm^2. After 24 h, DMSO or Avn C (100 and 200 μM) were added into culture media and incubated at 37°C & 5% CO_2 for 48 h. Cells were then washed two times with serum-free DMEM and hydrogen peroxide solution (H_2O_2) (Sigma-Aldrich, Cat #: 216763) was added to serum-free DMEM media at a final concentration of 200 μM with incubation at 37°C in a humidified atmosphere with 5% CO_2 for 1 h. Three biological replicates were conducted.

3.8.2 Cell treatment for anti-inflammatory activity assays

2DD cells were seeded at a density of 3,000 cells/cm^2. To evaluate anti-inflammatory activity of Avn C on 2DD cells, three different treatment conditions were applied: 1) Non-inducing conditions: cells were treated with DMSO or Avn C (100 and 200 μM diluted from 50mM Avn C stock solution) for 24 h; 2) H_2O_2-induced conditions: cells were treated with DMSO or Avn C (100 and 200 μM diluted from 50mM Avn C stock solution) for 48 h, followed by washing with serum-free DMEM. Hydrogen peroxide in serum-free media was added to achieve a final concentration of 200 μM with incubation as above for 1 h to induce cellular stress; and 3) TNFα-induced condition: cells were treated with DMSO or Avn C (100 and 200 μM diluted from 50mM Avn C stock solution) for 24 hours. TNFα was then added to media to a final concentration of 10 ng/mL and incubated for 4 h to induce the expression of pro-inflammatory markers. Three biological replicates were conducted.

3.8.3 Cell treatment for anti-proliferative effect assays

2DD cells and four cancer cells were seeded at a density of 3000 cells/cm^2. After 24 h, DMSO or Avn C (50, 100 and 200 μM diluted from 50mM Avn C stock solution) were added into culture media and incubated for 48 h. Three biological replicates were conducted.

3.9 Cell counts and cell viability

2DD cells and cancer cells were grown in 6-well plates under the previously described treatment conditions (3.8.3). At the end of the incubation period, cells were dissociated from the plate surface using TrypLE Express (Life Technologies, Carlsbad, CA, USA, Cat #: 12604013) and centrifuged at 200 relative centrifugal force (rcf) for 5 min to pellet cells. Cells were then re-suspended in culture media. Cell counts were conducted using 10 µL of cell suspension on a 0.0025 mm^2 Neubauer improved haemocytometer. To count and calculate total cell number, one drop of cell suspension was placed on the hemocytometer and then covered with a piece of cover glass. Cell numbers in five of the nine squares were counted. Total cell number was calculated as follows:

$$\text{Total number of cells} = \frac{\text{Number of cells counted}}{5} \times \text{volume of cell suspension (mL)} \times 10{,}000$$

Population doubling time was calculated as follows:

$$\text{Population doubling time (h)} = \text{Incubation time (h)} \times \frac{\ln(2)}{\ln(\frac{Final\ cell\ numbers}{Initial\ cell\ numbers})}$$

For cell viability test, cell suspensions were mixed 1:1 with 0.4% (v:v) trypan blue dye (VWR International, Cat #: CA97063-702). Total stained and unstained cells were counted by a haemocytometer under light microscope. Cells stained dark blue were considered not viable. Percentage of viable cells was calculated as follows:

$$\%\ \text{Viable cells} = \frac{\text{Total cell numbers} - \text{nonviable cell numbers}}{\text{Total cell numbers}} \times 100\%$$

3.10 Immuno-labeling of Ki67

2DD cells were grown on sterilized 22 mm^2 glass coverslips in 6-well dishes under the previously described conditions (3.8.3). At the end of the incubation period, cells were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4; pH 7.4) for 10 min at room temperature, followed by incubation in ice-old methanol/acetone (1:1 mixture) for 8 min at 4°C. Cells were washed two times with PBS and then permeabilized with 0.5% (v:v) Triton X-100/PBS for 10 min at room temperature. Cells were blocked with 1% (v:v) bovine serum albumin (BSA) for 1 h at room temperature. Cells were

placed on coverslips and were incubated in rabbit anti-Ki67 (1:2000 dilution; Novacastra, Newcastle, UK, Cat #: NCL-Ki67) diluted in 1.0% (v:v) BSA/PBS for 1 h in a dark humidity chamber at room temperature. Cells were then washed two times with PBS (1 mL per wash) and incubated in goat anti-rabbit A488 (1:200 dilution; Stratech/Jackson Scientific, UK, Cat #: 111545-003-JIR) diluted in 1.0% (v:v) BSA/PBS for 1 h in a dark humidity chamber. Nuclei were counterstained with VECTASHIELD® Mounting Medium with DAPI (4′,6-diamidino-2-phenylindole) (Vector Laboratories, Burlingame, CA, USA, Cat #: H1200) onto glass slides and sealed with nail varnish. A Nikon Y-IDP microscope fitted with an X-Cite fluorescence light source and a Nikon Digital Sight DS-U3 camera was used to collect images. Images were collected at 40X magnification with a constant exposure time. Images were imported into ImageJ (https://imagej.nih.gov/ij/), an image processing software. An arbitrary threshold was selected and any nuclei with a level above this value was considered positive and those below this threshold were considered negative. Percentage of Ki67 positive cells was calculated. Three biological replicates were conducted.

3.11 MitotrackerTM Orange labeling of intracellular free radicals

MitoTracker™ Orange CM-H_2TMRos (Molecular Probe, Eugene, OR, USA, Cat #: M-7511) is a reduced, non-fluorescent dye that stains mitochondria in live cells and becomes fluorescent when oxidized. 2DD cells were grown on sterilized 22 mm^2 glass coverslips in 6-well dishes under the previously described conditions (3.8.1). Coverslips with adhered cells were washed two times with serum-free media (2ml per wash). MitoTracker Orange was dissolved in DMSO and added to the serum-free media at a final concentration of 500 nM. After incubating for 30 min, cells on coverslips were fixed and image analysis was conducted as outlined above (3.10). Three biological replicates were conducted.

3.12 RNA extraction

2DD cells were grown in 10 cm diameter plates under the previously described treatment conditions (3.8.1, 3.8.2 and 3.8.3). RNA was extracted using TRIzol™ reagent (Life Technologies, Cat #: 15596026). Briefly, cells were dissociated from the surface of culture dishes using TrypLE Express (Life Technologies, Cat #: 12604013) and centrifuged at 200 x g for 5 min to pellet cells.

Cells were then re-suspended in 1 mL of TRIzol reagent and incubated at room temperature for 10 min. Two hundred microliters of chloroform was added and vigorously vortexed. Samples were then centrifuged at 12,000 rcf for 5 min at 4°C. The clear upper-phase was separated from the sample and placed in a new 1.5 mL centrifuge tube. RNA was precipitated by adding 1/10 sample volume of 3 M sodium acetate (pH 5.2) and 1X sample volume of isopropanol followed by incubation on ice for 30 min. Samples were centrifuged at 12,000 rcf for 30 min at 4°C. The supernatant was removed, and the resulting pellet of RNA was washed with 75% (v:v) cold ethanol. The pellet was air-dried and re-suspended in nuclease-free H_2O (Invitrogen, Cat #: 10977-015). Samples were then treated with DNase I (Roche, Mannheim, Germany, Cat #: 04716728001) at 37°C for 20 min to remove any DNA residues. Same sample volume of acid-phenol:chloroform (5:1, pH 4.5) (Life Technologies, Carlsbad, CA, USA, Cat #: AM9720) was added to samples and centrifuged at 12,000 rcf for 5 min at 4°C and the clear upper-phase was separated from the sample. RNA was precipitated by adding 1/10 sample volume of 3 M sodium acetate (pH 5.2) and 2.5 times sample volume of ethanol followed by incubation overnight at -20°C. Samples were centrifuged at 12,000 rcf for 30 min at 4°C and re-suspend in nuclease-free water. RNA concentration was determined using a NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and aliquots stored at -80°C. The quality of RNA samples was determined by sample analysis on ~~a~~ 1% agarose gel. RNA samples that showed sharp and clear 28S and 18S ribosomal RNA (rRNA) bands were used for cDNA synthesis. A representative gel is shown in Figure 3.1 with the 18S and 28S rRNA bands being both sharp and clear.

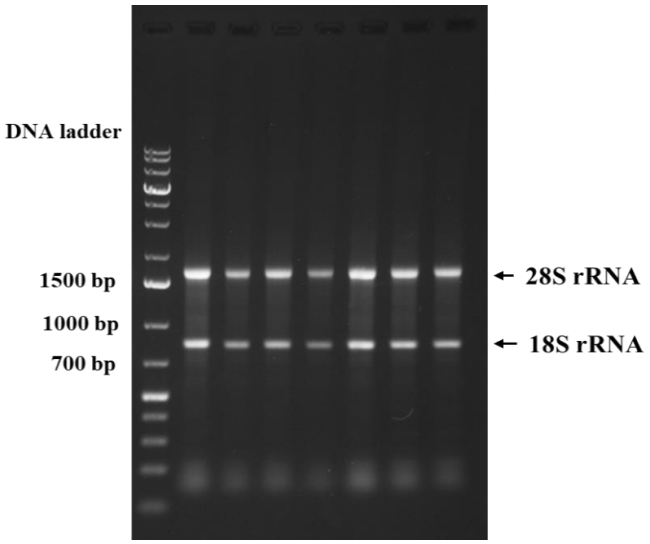


Figure 3.1 Gel electrophoresis of total RNA samples

3.13 cDNA synthesis

Two milligrams of RNA samples from 3.10 were used in complementary DNA (cDNA) synthesis reactions in combination with random hexamers and SuperScript™ III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA, Cat #: 18080044). In brief, 2 μg total RNA, 1 μL of 50 ng/μL random hexamers, 1 μL 10 mM of each dATP, dGTP, dCTP, dTTP were mixed and made up to 13 μL with nuclease free H_2O. The mixture was incubated for 5 min at 65°C. A master mix of 4 μL 5X First-Strand Buffer, 1 μL 0.1 M DTT, 1 μL RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen, Cat #: 10777019) and 1 μL of SuperScript™ III Reverse Transcriptase (200 U/μL) was added to a total reaction volume of 20 μL. Samples were incubated at 25°C for 5 min, 50°C for 60 min and 70°C for 15 min. Synthesized cDNA was diluted to a total volume of 200 μL with nuclease-free water and stored at -20°C until analysis.

3.14 Quantitative polymerase chain reaction (qPCR)

qPCR was performed following cDNA synthesis (3.10). A series of 10 μL reactions were set up using 5 μL PerfeCTa® SYBR® Green SuperMix for iQ (Quantabio, Beverly, MA, USA, Cat #: 95053-500), 2 μL cDNA template, 2 μL nuclease-free H_2O and 1 μL of 3 μM of each forward and reverse primers. qPCR reactions were conducted using a Rotor-Gene® Q qPCR (Qiagen, Germantown, MD, USA). All reactions for each gene were run in triplicate with non-template controls. Melt curve analyses were conducted to confirm the presence of single products in each reaction. Results were quantified using the ΔΔCt method against two normalizing genes: *SPARC* and *FKBP10*. The fold change values were calculated as follows:

$\Delta Ct_{\text{treatment sample}} = Ct_{\text{target gene}}$ in treatment sample - $Ct_{\text{normalizing gene}}$ in treatment sample

$\Delta Ct_{\text{control sample}} = Ct_{\text{target gene}}$ in control sample - $Ct_{\text{normalizing gene}}$ in control sample

$\Delta\Delta Ct = \Delta Ct_{\text{treatment sample}} - \Delta Ct_{\text{control sample}}$

Fold change = $2^{-\Delta\Delta Ct}$

The sequences of the primers used for qPCR were designed by Primer 3 (version 0.4.0; http://bioinfo.ut.ee/primer3-0.4.0) software and are listed in Table 3.1. qPCR data for each gene of interest were obtained from three independent biological replicates.

Table 3.2 Primers for RT-qPCR

Gene	Forward primer (5'→3')	Reverse primer (5'→3')	Amplicon (bp)
Normalizing genes			
SPARC	TACATCGGGCCTTGCAAATAC	GGTGACCAGGACGTTCTTGAG	99
FKBP10	GCCGTGCTAATCTTCAACGTC	GGTGGTCTCATTGCAGGTCTC	105
Antioxidant enzyme genes			
GSS	AGCTTTCCATCTGAGGACCAG	TCCTATCCCAAGTCAGGCACT	188
HMOX1	AAAGGAGGAAGGAGCCTATGG	AGCTGCCACATTAGGGTGTCT	149
SOD1	GGCAAAGGTGGAAATGAAGAA	GGGCCTCAGACTACATCCAAG	112
GPx1	CCTCCCCTTACAGTGCTTGTT	GAGAAGGCATACACCGACTGG	115
CAT	TGCAAGCTAGTGGCTTCAAAA	TCCAATCATCCGTCAAAACAA	143
Pro-inflammatory cytokine genes			
IL-1β	GCTACGAATCTCCGACCACCA	AACCAGCATCTTCCTCAGCTTG	91
IL-6	CGTCCGTAGTTTCCTTCTAGCTT	CAAAGGAGGACCTTGTGGCA	103
IL-8	TGCAGTTTTGCCAAGGAGTG	TGATAAATTTGGGGTGGAAAGG	83
TNFα	CAATGGCGTGGAGCTGAGAG	TCTGGTAGGAGACGGCGATG	152

3.15 Protein extraction and quantification

2DD cells were grown in 10 cm diameter plates under the previously described treatment conditions (3.8.1, 3.8.2 and 3.8.3). At the end of the incubation period, cells were dissociated from the surface of culture plates using TrypLE Express (Life Technologies) and centrifuged at 200 x g for 5 min to pellet cells. Supernatant was removed followed by cell re-suspention in RIPA buffer (150 mM NaCl, 25 mM Tris-HCl pH 7.6, 1.0% w/v sodium deoxycholate, 1 mM EDTA pH 8.0 and 1.0% v/v NP-40) or Laemmli lysis buffer (62.5 mM Tris-HCl pH 6.8, 2% w:v SDS, 10% v/v glycerol and 5% v/v 2-mercaptoethanol) containing Protease Inhibitor Cocktail 2 (Sigma-Aldrich, Cat #: P8340) and Phosphatase Inhibitor Cocktail 2 (Sigma-Aldrich, Cat #: P5726) at a ratio of 100:1:1. Samples were incubated on ice for 30 min with intermittent vortexing. All protein extracts were stored at -20°C until analyses.

If RIPA buffer was used for protein extraction, protein extracts were quantified by Bradford assay using Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories,

Mississauga, ON, Cat #: 500-0006). Briefly, Dye Reagent Concentrate was diluted 1:5 with Milli-Q™ water. For each sample, 2 µL cell lysate was diluted in 18 µL Milli-Q™ water and then added into 1.0 mL diluted Bio-Rad dye reagent. Following incubation at room temperature for 15 minutes, absorbance was read at 595 nm. BSA was used as a standard at the concentrations of 0.2, 0.4, 0.6, 0.8 mg/mL diluted in Milli-Q™ water. The absorbance of 1 mL diluted dye reagent containing different BSA concentrations was obtained and a standard curve was plotted. The blank consisted of 20 µL of Milli-Q™ water and 1 mL diluted dye reagent. The concentration of each protein extract was calculated against the standard curve.

If Laemmli lysis buffer was used for protein extraction, protein extracts were quantified by UV spectroscopy using a NanoDrop™ 2000 Spectrophotometer. 1.5 µL cell lysate was loaded and protein concentration was measured at 280 nm. Laemmli lysis buffer (1.5 µL) was used as the blank.

3.16 Western blot

All protein samples were diluted to final concentrations of 1.0 or 1.5 µg/µL. For protein extracts prepared in RIPA buffer, samples were diluted in RIPA buffer and SDS-PAGE Loading Buffer (5X: 250 mM Tris-HCl pH 6.8, 30% v/v glycerol, 0.02% w/v bromophenol blue, 20% w/v 2-mercaptoethanol and 10% w/v SDS). For protein extracts prepared in Laemmli lysis buffer, samples were diluted in 1X Laemmli lysis buffer and 1 µL of 1% bromophenol (w:v) blue was added to each sample. All diluted protein extracts were denatured by boiling at 95°C for 5 min and then cooled on ice. Samples were then centrifuged for 10 min at 15,000 rpm, and the supernatants were transferred to new labelled tubes and stored at -20°C until analyses.

Denatured protein samples (15 to 30 µg cell lysate per sample) were loaded and separated on a polyacrylamide gel with a 5.0% polyacrylamide stacking top gel and a resolving bottom gel in 1X running buffer (25 mM Tris base, 192 mM glycine and 0.1% SDS) at 125 V. Proteins were then transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Cat #: 1620115) in 1X transfer buffer (25 mM Tris base, 192 mM glycine and 20% methanol) using Trans-Blot® SD semi-dry transfer cell (Bio-Rad Laboratories) at 25 V. The membranes were then blocked in 5.0% skim milk powder in phosphate-buffered saline with 0.05% Tween® 20 (PBST) for 1 h at room temperature, followed by incubating overnight at 4°C in primary antibody diluted in 5.0% skim

milk powder in PBST. Primary antibodies used were rabbit anti-phospho-NF-κB p65 (Ser536) antibody (1:1000 dilution; Cell Signaling Technology, Danvers, MA, USA, Cat #: 3033), mouse anti-SIRT1 antibody (1:1000 dilution; Abcam, Cat #: ab110304), mouse anti-phospho-mTOR (Ser 2448) antibody (1:500 dilution; Santa Cruz, Dallas, TX, USA, Cat #: sc-293133), rabbit anti-LC3II (1:1000 dilution; Abcam, Cat #: ab48394), mouse anti-heme oxygenase 1 antibody (1:500 dilution; Santa Cruz, Cat #: sc-136960) mouse anti p-Histone H2A.X (Ser 139) antibody (1:500 dilution; Santa Cruz, Cat #: sc-517348) and rabbit anti-β-actin (1:2000 dilution; Abcam, Cat #: ab8227). Following primary antibody incubation, membranes were washed three times in 5.0% skim milk/PBST for 10 min. Membranes were then incubated in secondary antibody diluted in 5.0% skim milk/PBST for 1 h at room temperature. Secondary antibodies used were goat anti-rabbit horse radish peroxidase (HRP) (1:2000 dilution; Abcam, Cat #: ab97069) or donkey anti-mouse HRP (1:2000 dilution, Jackson Scientific, West Grove, PA, USA, Cat #: 715-035-150). Membranes were then washed two times in 5.0% skim milk/PBST for 10 min ~~each~~ and two times in PBST for 5 min. Protein bands were visualized with chemiluminescence using enhanced chemiluminescence (ECL) reagent (100 mM Tris-HCl pH 8.5, 0.2 mM *p*-coumaric acid, 1.25 mM 3-aminophtalhydrazide and 0.1% v/v H_2O_2). β-actin was used as a loading control.

3.17 Chromatin immunoprecipitation-qPCR (ChIP-qPCR)

2DD cells were grown in 15 cm diameter plates and treated with DMSO (control) or 100 µM Avn C for 48 h. At the end of the treatment period, cells were fixed with 1% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA, Cat #: 15714) in 15 mL serum-free culture media per plate for 10 min at room temperature. Fixation was terminated by adding glycine into culture media to a final concentration of 125 mM followed by incubation at room temperature for 10 min. Media was then removed and cells were washed two times with ice-cold PBS. Cells were scraped in ice-cold PBS and centrifuged at 200 rcf for 5 min at 4°C to pellet cells. Cells were then re-suspended in 400 µL ChIP lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.0) containing Protease Inhibitor Cocktail 2 (Sigma-Aldrich, Cat #: P8340) and Phosphatase Inhibitor Cocktail 2 (Sigma-Aldrich, Cat #: P5726). Following 10 min of incubation on ice, cells were sonicated on ice at ~7% power output and 30% duty cycle for 1 min. Samples were then centrifuged at 12,000 rcf 4°C for 10 min and supernatants were transferred to new 1.5 mL tubes as sheared chromatin samples. Aliquots of chromatin samples were stored at -80°C until analyses.

From each treatment condition, 30 µL of the chromatin sample was used as the input sample and 60 µL of the chromatin sample was used for each immunoprecipitation. A 2.5 µg aliquot of mouse anti-Nrf2 antibody or 2.5 µg of rabbit anti-phospho-NF-κB p65 (Ser536) antibody was added to 60 µL chromatin sample diluted 10 times in ChIP buffer (0.01% SDS, 1.1% Triton X100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.0 and 167 mM NaCl) containing Protease Inhibitor Cocktail 2 (Sigma-Aldrich, #: P8340) and Phosphatase Inhibitor Cocktail 2 (Sigma-Aldrich, Cat #: P5726). 2.5 µg donkey anti-mouse HRP was used as the non-specific antibody control. The mixture was incubated at 4°C overnight with rotation, followed by binding to 50 µL Dynabeads™ Protein A (Life Technologies, Oslo, Norway, Cat #: 10006D) at 4°C for 1 h. The samples were then washed three times with ChIP washing buffer I (0.1% SDS, 1% Triton X100, 2 mM EDTA, 20 mM Tris pH 8.0 and 150 mM NaCl) for 5 min each, three times with ChIP washing buffer II (0.1% SDS, 1% Triton X100, 2 mM EDTA, 20 mM Tris pH 8.0 and 500 mM NaCl) for 5 min each and three times with ChIP washing buffer III (1 mM EDTA and 10 mM Tris-HCl pH 8.0) for 5 min each. After washing steps, samples were eluted with 500 µL freshly made elution buffer (1% SDS and 0.1 M $NaHCO_3$) for 1 h at room temperature. For each eluted and input sample, crosslinks were reversed by adding 200 mM NaCl, 12.5 mM EDTA and 2 µL proteinase K (Invitrogen, Cat #: 25530049), followed by incubation at 65°C for 5 h with agitation at 900 rpm in a thermal mixer. The DNA from each sample was extracted by phenol-chloroform as follows: after reverse-crosslinking, 500 µL of phenol-chloroform (1:1, pH 8.0) was added into each tube and vigorously vortexed. Samples were then centrifuged at 12,000 rcf for 10 min at 4°C. The clear upper-phase was separated from the sample and placed in a new 1.5 mL centrifuge tube. DNA was precipitated by adding 2.0 µL glycogen and 1X sample volume of isopropanol. Samples were then centrifuged at 12,000 rcf for 30 min at 4 °C, and the supernatant was removed. The DNA pellet from the input sample was re-suspended in 40 µL nuclease-free water and DNA from immunoprecipitated sample was re-suspended in 80 µL nuclease-free water. Chromatin shearing efficiency was checked monitored by running 5 µL input DNA samples on a 1.5% agarose gel and most DNA fragments were found between 200 and 1000 bp on the gel. A representative gel picture is shown in Figure 3.2 that indicates sheared DNA fragment sizes after sonication.

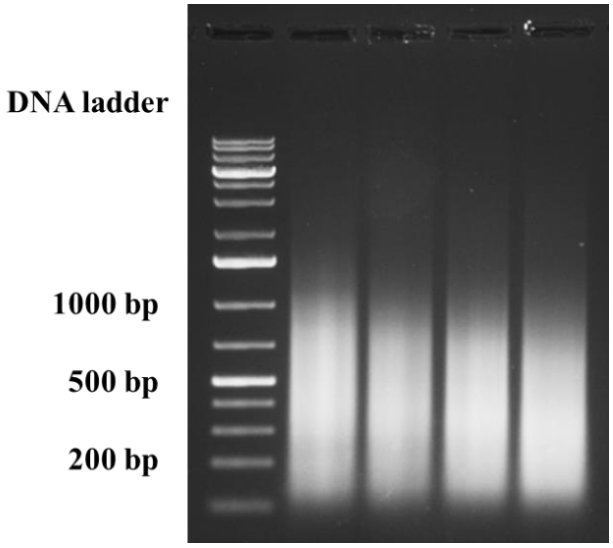


Figure 3.2 Gel electrophoresis of sheared DNA samples for ChIP assay

qPCR was performed following DNA extraction. Ten microliter reactions were set up using 5 μL PerfeCTa® SYBR® Green SuperMix for iQ (Quantabio, Beverly, MA, USA, Cat #: 95053-500), 1 μL ChIP DNA sample, 3 μL H_2O and 1 μL of 3 μM forward and reverse ChIP primers. qPCR reactions were conducted using a Rotor-Gene® Q qPCR (Qiagen, Foster City, CA, USA). All reactions for each gene were run in triplicate with non-template controls. ChIP-qPCR data was normalized by the percent input method. Standard error was calculated as a function of the standard deviations of triplicates. ChIP-qPCR primers used are listed in Table 3.2.

Table 3.3 ChIP-qPCR primers

Gene	Forward primer (5'→3')	Reverse primer (5'→3')	Amplicon (bp)
HMOX1	CCGCCCCGAGATCTGTTTTC	ATGTCCCGACTCCAGACTCC	157
NQO1	TGAGAGTCCTGGGGAGACAT	CACCCAGGGAAGTGTGTTGT	103
IL-1β	TTGCCCTTCCATGAACCAGAG	AAGCAGAAGTAGGAGGCTGAGA	147
IL-8	GTGATGACTCAGGTTTGCCCT	CTTATGGAGTGCTCCGGTGG	139

3.18 Statistical analysis

Results were presented as mean ± standard deviation of three biological replicates. Two-tailed Student's *t* test or one-way ANOVA with Tukey's HSD (honest significant difference) was

performed to assess statistical significance between groups as indicated in the legends. p-values <0.05 were considered statistically significant.

4.0 RESULTS

4.1 Identification and quantitative analysis of Avn A, B & C in oat seeds by HPLC-PDA

To identify the presence and concentrations of Avn A, Avn B and Avn C, methanol extracts of oat seeds from ten oat varieties and breeding lines were analyzed by HPLC-PDA. Avn A, B and C in oat extracts were identified based on their retention times and UV-visible spectral profiles compared with pure standards (Figure 4.1). Figure 4.2 shows a representative HPLC chromatogram of the oat extract from CDC Dancer. The three peaks labeled 1, 2 and 3 in the chromatogram were identified as Avn C, Avn A and Avn B. All ten oat extracts showed these peaks clearly, which indicates that Avn A, B and C can be successfully extracted using the methods described above (3.2 and 3.3).

Concentrations of Avn A, B and C in oat extracts were determined by comparing the peak area of the identified Avn A, B and C in the sample with standard curves from the three standards (concentration vs. peak area). As shown in Table 4.1, the concentrations of Avn A, B and C present in the ten oat samples ranged from 1.8 to 21.4, 2.2 to 34.7 and 4.4 to 34.5 mg/kg, respectively, and the total amount of Avn A, B and C in the ten oat extracts ranged from 8.4 to 90.6 mg/kg. Among the ten samples, BW10 not only contained the highest total amount of the three avenanthramides (90.6 ± 2.3 mg/kg), it also showed the highest concentrations of individual Avn A (21.4 ± 0.7 mg/kg), Avn B (34.7 ± 0.9 mg/kg) and Avn C (34.5 ± 0.9 mg/kg). In contrast, CDC Sol-Fi contained the lowest total Avns (A+B+C) of 8.4 ± 0.3 mg/kg, which was ~11 times less than that observed in BW10. It also showed the lowest concentrations of individual Avn A (1.7 ± 0.1 mg/kg), Avn B (2.2 ± 0.1 mg/kg) and Avn C (4.4 ± 0.2 mg/kg).

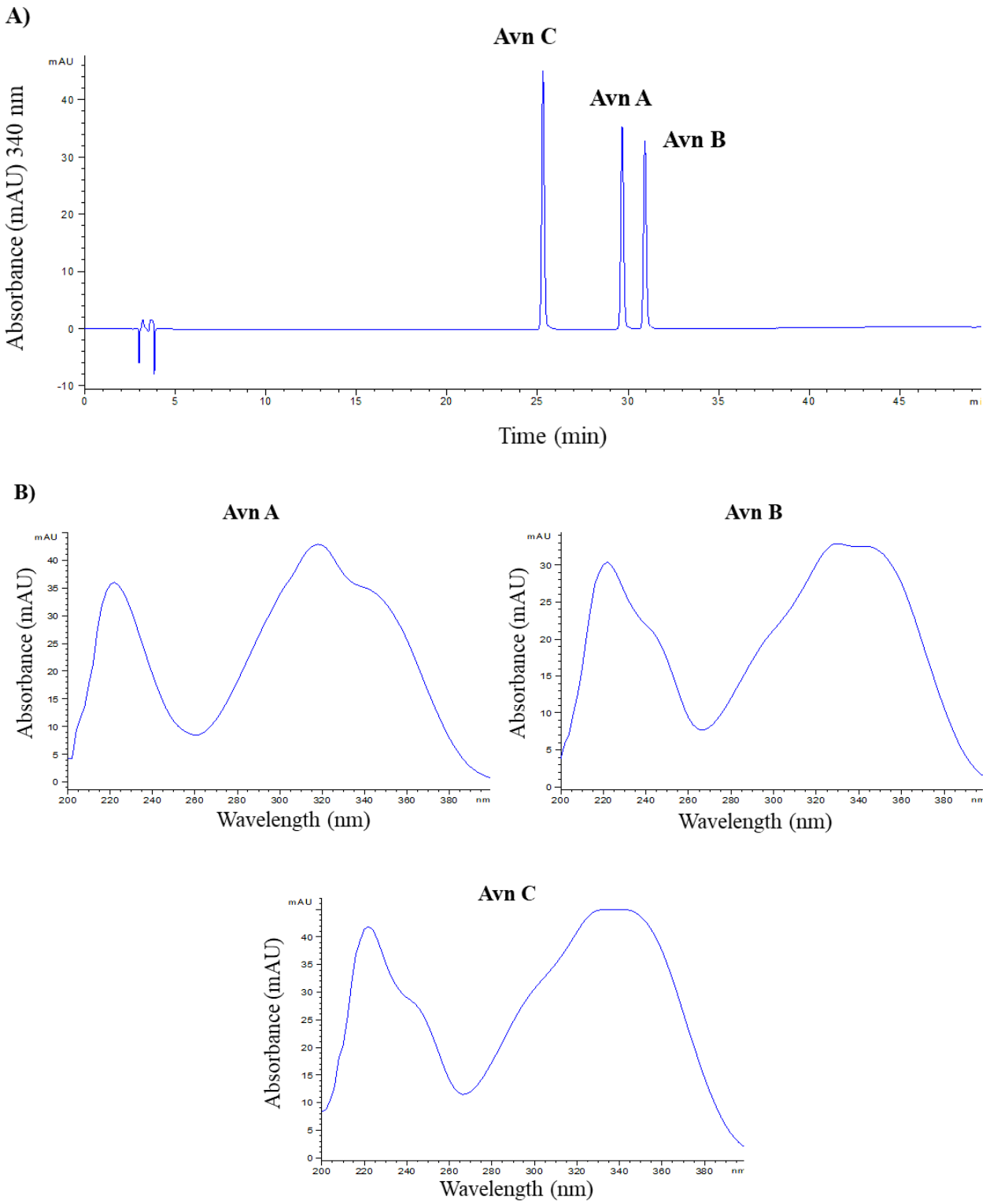


Figure 4.1 HPLC-PDA chromatogram (A) and UV-visible spectral profiles (B) of Avn A, B and C standards (10 ppm) monitored at 340 nm.

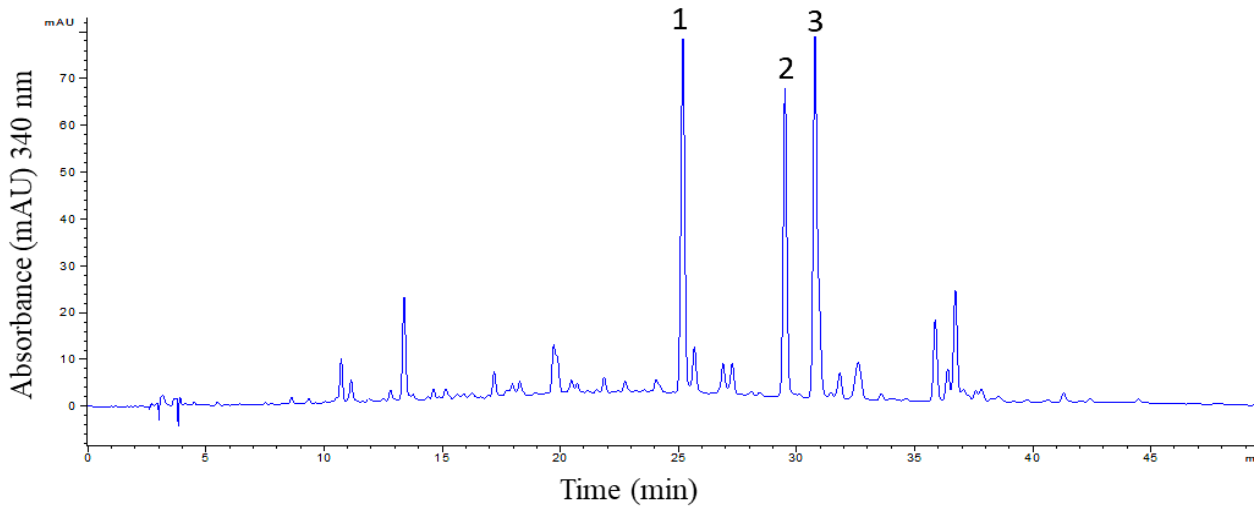


Figure 4.2 HPLC-PDA chromatogram of the oat extract from the CDC Dancer variety monitored at 340 nm. Peak assignments: 1. Avn C; 2. Avn A; 3. Avn B

Table 4.1 Concentrations (mg/kg) of individual and total Avn A, B and C in oat seeds of ten varieties and breeding lines.

Variety	Avn A	Avn B	Avn C	Total
BW10	21.4 ± 0.7^{a}	34.7 ± 0.9^{a}	34.5 ± 0.9^{a}	90.6 ± 2.3^{a}
BW513	5.4 ± 0.2^{b}	9.3 ± 0.1^{b}	11.0 ± 0.1^{b}	25.8 ± 0.3^{b}
BW5303	3.8 ± 0.1^{c}	4.4 ± 0.1^{c}	6.6 ± 0.1^{c}	14.8 ± 0.1^{c}
CDC Dancer	19.1 ± 0.2^{d}	19.6 ± 0.3^{d}	19.0 ± 0.2^{d}	57.6 ± 0.2^{d}
CDC Morrison	8.0 ± 0.3^{e}	11.4 ± 0.4^{e}	10.3 ± 0.2^{e}	29.6 ± 0.9^{e}
CDC Sol-Fi	1.8 ± 0.1^{f}	2.2 ± 0.1^{f}	4.4 ± 0.2^{f}	8.4 ± 0.3^{f}
Jumbo	3.9 ± 0.3^{c}	4.3 ± 0.1^{c}	4.5 ± 0.1^{fg}	12.7 ± 0.5^{g}
Marion	2.2 ± 0.2^{g}	3.1 ± 0.2^{g}	4.8 ± 0.1^{g}	10.0 ± 0.5^{h}
Newburg	2.8 ± 0.2^{h}	4.8 ± 0.1^{h}	5.5 ± 0.1^{h}	13.1 ± 0.3^{g}
OT2021	9.8 ± 0.2^{i}	12.6 ± 0.3^{i}	11.5 ± 0.2^{i}	33.9 ± 0.6^{i}

Note: Values represent mean ± standard deviation of three replicates. Values in the same column followed by a common letter were not statistically different ($p<0.05$; ANOVA/Tukey) between varieties.

4.2 Total phenolic content and antioxidant activities of oat phenolic-rich extracts

4.2.1 Total phenolic content determined by the Folin-Ciocalteau assay

Total phenolic content of oat extracts from the ten oat varieties and breeding lines were determined by Folin-Ciocalteu (FC) method, which measures the absorption reduction of a phosphomolybdic-phosphotungstic acid reagent (FCR) to a blue-colored complex under alkaline conditions in the presence of phenolics. Under basic conditions, dissociation of a phenolic proton leads to a phenolate anion, which is capable of reducing FCR (Singleton *et al.*, 1999). Gallic acid was used as a standard and results were reported as gallic acid equivalent (GAE) per 100 g oat powder. As shown in Table 4.2, total phenolic content of the ten oat extracts ranged from 78.5 ± 0.7 to 121.2 ± 2.5 GAE/100 g. The highest total phenolic content was found in BW10 (121.2 ± 2.5 GAE/100 g), followed by OT 2021 (102.5 ± 2.0 GAE/100 g) and BW5303 (97.9 ± 1.1 GAE/100 g), and the lowest was found in the Newburg variety (78.5 ± 0.7 GAE/100 g).

Table 4.2 Total phenolic content of ten oat phenolic extracts from different varieties as determined by the Folin-Ciocalteau assay

Variety	Total phenolic content (mg GAE/100 g oat)
BW 10	121.2 ± 2.5^{a}
BW 513	87.7 ± 1.2^{b}
BW 5303	97.9 ± 1.1^{c}
CDC Dancer	86.8 ± 1.5^{bd}
CDC Morrison	84.5 ± 1.3^{de}
CDC Sol-Fi	82.3 ± 1.4^{e}
Jumbo	89.7 ± 1.5^{b}
Marion	89.2 ± 1.4^{b}
Newburg	78.5 ± 0.7^{f}
OT 2021	102.5 ± 2.0^{g}

Note: Values represent mean ± standard deviation of three replicates. Values followed by a common letter were not statistically different ($p<0.05$; ANOVA/Tukey) between varieties. GAE: gallic acid equivalent.

4.2.2 ABTS & DPPH radical scavenging activity

ABTS and DPPH free radical systems were used to determine free the radical scavenging activity of ten oat extracts. Both assays measure the reduction in colour of the synthetic free radical solutions caused by electron transfer from antioxidants (Huang *et al.* 2005; Sharma and Singh, 2013). Specifically, the ABTS assay is based on the generation of a blue/green $ABTS^{\bullet+}$ radical cation that can be reduced by antioxidants; whereas the DPPH assay is based on the reduction of the purple $DPPH^{\bullet}$ radical to 1,1-diphenyl-2-picryl hydrazine in the presence of antioxidants (Huang *et al.* 2005; Sharma and Singh, 2013). By measuring the changes of colour intensity in the sample solution, free radical scavenging activities of oat extracts were determined. Results are presented as concentrations (g oat/mL) required to inhibit 50% of the free radicals in solution (IC_{50}) (Table 4.3). A low IC_{50} value represents a higher free radical scavenging activity. The highest ABTS free radical scavenging activity was found in BW10 with an IC_{50} value of 0.87 ± 0.02 g oat/mL, whereas the lowest ABTS free radical scavenging activities were found in BW513 and Newburg with an equal IC_{50} value of 1.38 ± 0.01 g oat/mL. The highest DPPH free radical scavenging activity was found in OT2021 with an IC_{50} value of 0.279 ± 0.007 g oat/mL, whereas the lowest DPPH free radical scavenging activity was found in Newburg with an IC_{50} value of 0.798 ± 0.049 g oat/mL.

Table 4.3 ABTS and DPPH free radical scavenging activities of ten oat phenolic extracts from different varieties.

Variety	ABTS IC_{50}	DPPH IC_{50}
BW 10	0.87 ± 0.02^{a}	0.29 ± 0.00^{a}
BW 513	1.38 ± 0.01^{b}	0.38 ± 0.01^{b}
BW 5303	1.16 ± 0.02^{c}	0.37 ± 0.01^{b}
CDC Dancer	1.03 ± 0.04^{df}	0.35 ± 0.02^{b}
CDC Morrison	1.06 ± 0.03^{d}	0.36 ± 0.01^{b}
CDC Sol-Fi	1.26 ± 0.03^{e}	0.38 ± 0.01^{b}
Jumbo	1.25 ± 0.02^{e}	0.46 ± 0.01^{c}
Marion	1.21 ± 0.05^{ce}	0.41 ± 0.00^{d}
Newburg	1.38 ± 0.01^{b}	0.80 ± 0.05^{e}
OT 2021	0.99 ± 0.02^{f}	0.28 ± 0.01^{f}

Note: Values represent mean ± standard deviation of three replicates. Values in the same column followed by a common letter were not statistically different ($p<0.05$; ANOVA/Tukey) between varieties. IC_{50}: concentration (g oat/mL) required for 50% free radical inhibition.

4.3 ABTS & DPPH radical scavenging activity of Avn A, B & C

Free radical scavenging activities of pure Avn A, B and C were determined by the same ABTS and DPPH free radical assays used for the oat extracts (4.2.2). Gallic acid, caffeic acid and Trolox were used as references for comparison. The concentrations required to inhibit 50% of the free radicals in solution (IC_{50}) are presented in Table 4.4. From these results, the order of free radical scavenging activity in the ABTS assay was, gallic acid > Avn C = Avn B > Trolox = caffeic acid = Avn A, whereas in the DPPH assay the order of free radical scavenging activity was gallic acid > Avn C > caffeic acid > Trolox > Avn B > Avn A. Collectively, Avn C not only showed the highest antioxidant activity among the three avenanthramides, but also showed a higher antioxidant activity than caffeic acid and Trolox, which are commonly considered as strong antioxidants.

Table 4.4 ABTS and DPPH free radical scavenging activities of Avn A, B and C as compared to Caffeic acid, Gallic acid and Trolox

	ABTS IC_{50}	**DPPH IC_{50}**
Avn A	1.20 ± 0.01^{a}	1.26 ± 0.03^{a}
Avn B	0.84 ± 0.02^{b}	0.61 ± 0.01^{b}
Avn C	0.82 ± 0.02^{b}	0.41 ± 0.01^{c}
Caffeic acid	1.19 ± 0.02^{a}	0.45 ± 0.03^{d}
Gallic acid	0.37 ± 0.01^{c}	0.14 ± 0.01^{e}
Trolox	1.18 ± 0.01^{a}	0.54 ± 0.02^{f}

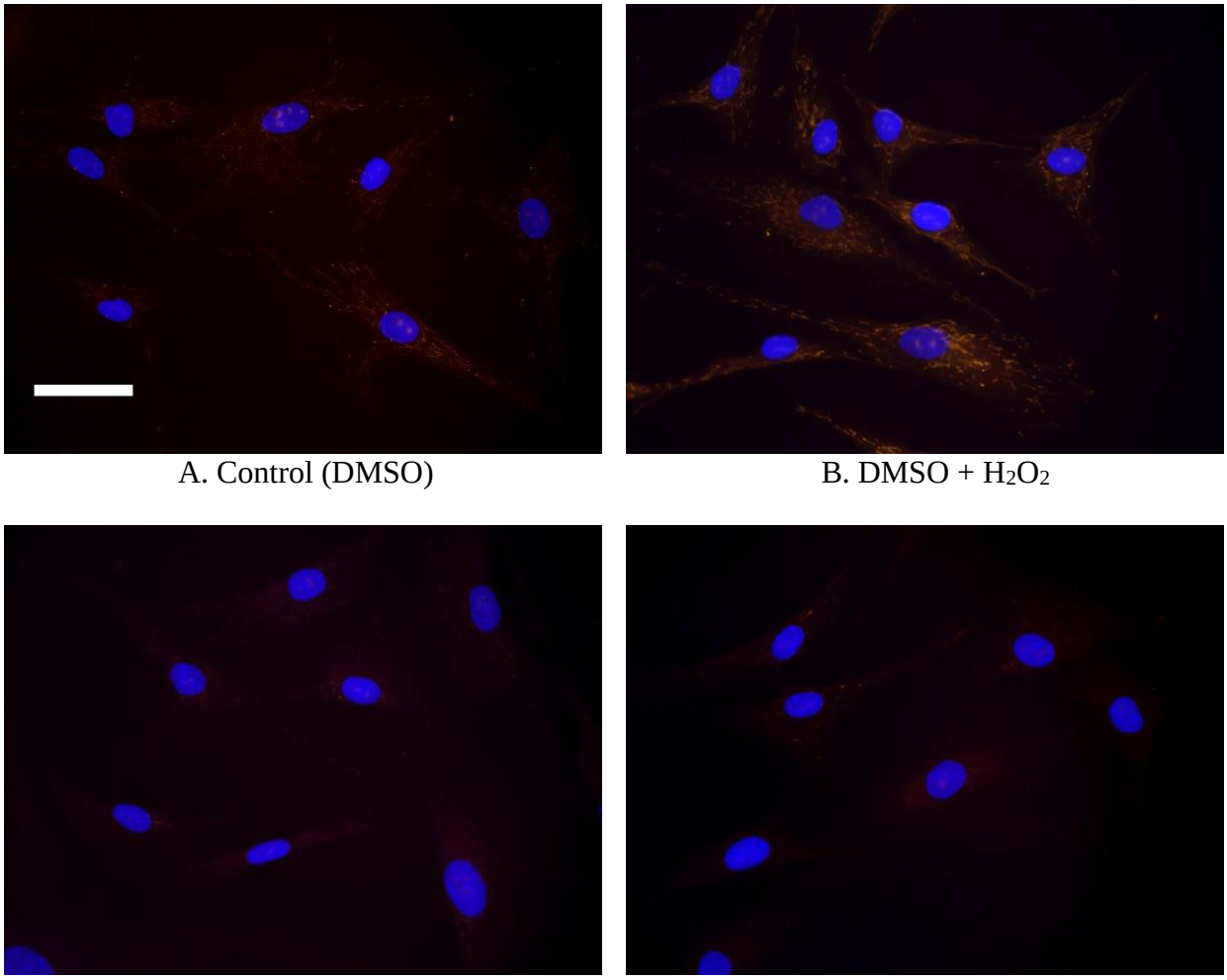
Note: Values represent mean ± standard deviation of three replicates. Values in the same column followed by a common letter were not statistically different ($p<0.05$; ANOVA/Tukey) between different compounds. IC_{50}: concentration (mM) required for 50% free radical inhibition.

4.4 Avn C protects normal human skin fibroblasts from H_2O_2-induced cellular damage

4.4.1 Avn C protects 2DD fibroblasts from H_2O_2-induced oxidative stress

Results from the ABTS and DPPH free radical scavenging assays demonstrated that Avn C had promising antioxidant potential; however, it is unclear how well this phenolic compound penetrates cell membranes and is able to scavenge free radicals in normal human cells. To evaluate cytoprotective effects of Avn C against H_2O_2-induced oxidative stress, normal human fibroblasts (2DD) were used as a cell culture model. 2DD cells were pre-treated with Avn C for 48 hours prior to 1 h exposure to H_2O_2 to induce oxidative stress. Free radicals inside cells were stained with a reduced, non-fluorescent dye MitoTracker Orange that becomes fluorescent when oxidized by free radicals. Fluorescence micrographs were collected and the intensity of the Mitotracker™ dye was examined. Figure 4.3 illustrates representative cell images stained with MitoTracker Orange (orange) for labeling free radicals and DAPI (blue) for labeling cell nuclei? Control cells (DMSO only) showed a small amount of orange fluorescent signal (Figure 4.3 A) and a dramatic increase of the signal was observed in the cells with H_2O_2 treatment alone (Figure 4.3 B). Pre-treatment with 100 and 200 μM Avn C for 48 h before H_2O_2 treatment showed decreased orange fluorescent signals (Figure 4.3 C&D) when compared with H_2O_2 treatment alone, indicating that Avn C penetrated into cells and was capable of scavenging intracellular free radicals.

To further evaluate the protective capacity of Avn C against H_2O_2-induced oxidative stress, we examined transcript levels of antioxidant genes normally stimulated by oxidative stress. After pre-treatment with Avn C for 48 h followed by 1 h H_2O_2 exposure, total RNA was extracted, converted to cDNA and evaluated by qRT-PCR. Hydrogen peroxide treatment without cell pre-treatment with Avn C resulted in a significant increase in the transcripts from the *GSS*, *HMOX1*, *SOD1*, *GPx1* and *CAT* genes (Figure 4.4). By comparison with H_2O_2 treatment alone, pre-treatment of 2DD cells with 200 μM Avn C significantly suppressed increased levels of all the transcripts induced by H_2O_2. Also, pre-treatment with 100 μM Avn C significantly suppressed increased levels of all the transcripts with the exception of *CAT*.



A. Control (DMSO)

B. DMSO + H_2O_2

C. 100 μM Avn C + H_2O_2

D. 200 μM Avn C + H_2O_2

Figure 4.3 Avn C reduces H_2O_2-induced intracellular free radicals in 2DD fibroblasts. 2DD primary dermal fibroblasts growing on coverslips were pre-treated with DMSO (control) or Avn C (100 μM and 200 μM; 48h) prior to 1 h exposure to 200 μM H_2O_2. Following the treatments, cells were washed and incubated with MitoTracker™ Orange dye, fixed and mounted for fluorescence microscopy. MitoTracker™ Orange dye reacts with free radicals inside cells to generate orange fluorescence. Cell chromatin is counterstained with DAPI (blue) to indicate nuclear location. Scale bar - 50 μm.

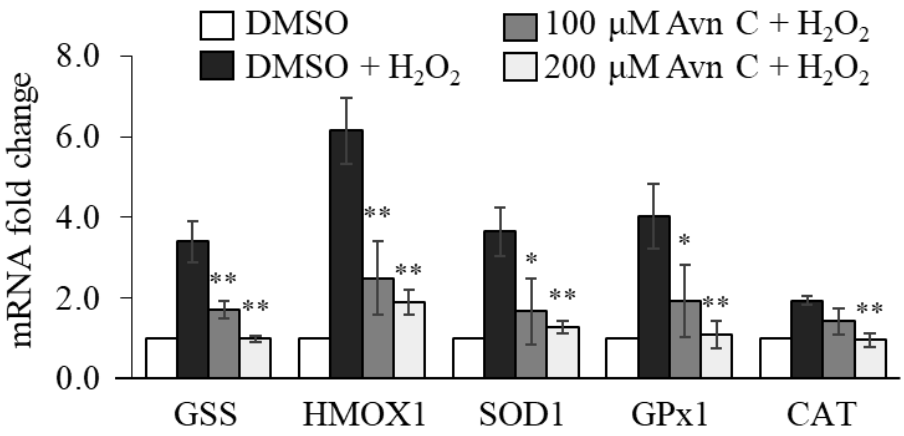


Figure 4.4 Avn C suppresses H_2O_2-induced mRNA expression of antioxidant enzymes. 2DD primary fibroblasts were pre-treated with DMSO (0) or Avn C (100 μM and 200 μM) for 48 h prior to exposure to 200 μM H_2O_2. qRT-PCR on cDNA libraries was performed for glutathione synthetase (*GSS*), heme oxygenase 1 (*HMOX1*), superoxide dismutase 1 (*SOD1*), glutathione superoxidase 1 (*GPx1*) and catalase (*CAT*) gene transcripts. Graphs indicate the average fold change from three biological replicates. Error bars represent the standard deviations. **p*-value <0.05, ***p*-value <0.01 vs H_2O_2 treatment alone

4.4.2 Avn C protects 2DD fibroblasts from H_2O_2-induced DNA damage

One of the major biological consequences of free radical production and exposure is increased DNA damage. Upon DNA damage, the histone variant H2AX-γ becomes phosphorylated (p-H2AXγ) and is deposited at the sites of damage, facilitating the recruitment of repair machinery; therefore, p-H2AXγ is commonly used as a sensitive marker for DNA double-strand breaks. We evaluated the ability of Avn C to protect cells from H_2O_2-induced DNA damage by measuring p-H2AXγ expression. After pre-treatment of normal human skin fibroblasts (2DD) with Avn C for 48 h followed by 1 h H_2O_2 exposure, a whole cell extract was obtained and subjected to western blot analysis. We observed that pre-treatment with Avn C (100 μM and 200 μM) significantly decreased levels of p-H2AXγ (as determined by γ-H2AX to β-actin ratios) induced by H_2O_2 (Figure 4.5), further demonstrating the cytoprotective effects of Avn C on normal cells.

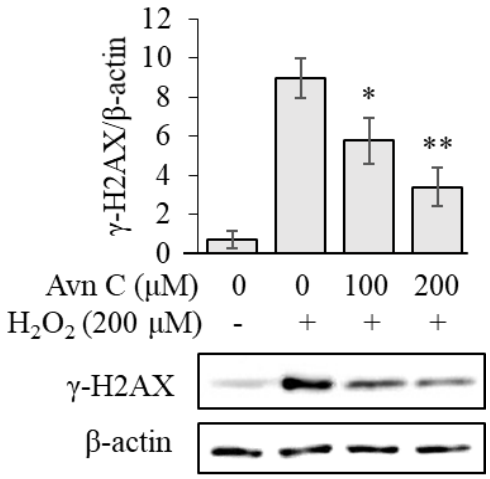


Figure 4.5 Avn C suppresses H_2O_2-induced DNA double-strand breaks. Normal human skin fibroblasts (2DD) were pre-treated with DMSO (0) or Avn C (100 μM and 200 μM) for 48 h followed by 1 h exposure to 200 μM H_2O_2. Whole cell extract was then obtained and subjected to western blot analysis. Data are presented as a ratio of γ-H2AX to β-actin. Representative blot images are shown. All graphs indicate mean values from three biological replicates. Error bars represent standard deviations. **p*-value <0.05, ***p*-value <0.01 vs H_2O_2 treatment alone.

4.4.3 Avn C protects 2DD fibroblasts from H_2O_2-induced inflammatory responses

Free radicals inside cells not only promote oxidative stress, they also increase inflammatory response (Mittal *et al.*, 2014; Morgan and Liu, 2011). As we observed that Avn C showed a cytoprotective effect against H_2O_2-induced oxidative stress in 2DD fibroblasts, we evaluated if Avn C could also reduce H_2O_2-induced inflammation in 2DD cells. We first measured the ability of Avn C to suppress the transcript levels of pro-inflammatory markers following H_2O_2 treatment. Transcripts from *Il-1β, Il-6, Il-8* and *TNF-α* genes were evaluated by qRT-PCR. Avn C significantly reduced the amounts of transcripts from *Il-6, Il-8* and *TNF-α* under pre-treatment conditions of 100 and 200 μM Avn C, whereas a significant reduction of *Il-1β* was only observed at the 200 μM pre-treatment concentration (Figure 4.6 A).

Evidence has shown that oxidative damage to cells leads to the release of TNF-*α* from these damaged cells (Kim *et al.*, 2008). Binding of TNF-*α* to cell receptors activates NF-*κ*B, a dimer of p65 (RelA) and p50 proteins, which results in the further generation of proinflammatory cytokines (Kim *et al.*, 2008). As we observed that Avn C suppressed H_2O_2-induced pro-inflammatory cytokine transcripts, we further evaluated if this suppression effect was mediated by inhibition of NF-κB activation in 2DD fibroblasts. Western blot analyses of phosphorylated NF-

κB p65 (Ser536) demonstrated that H_2O_2 treatment alone resulted in an increased protein level of phosphorylated p65, which was significantly reduced by 100 and 200 μM Avn C pre-treatment (Figure 4.6 B). These observations indicate that Avn C is able to protect normal human skin fibroblasts against H_2O_2-induced inflammation through both the suppression of increased levels of pro-inflammatory cytokine transcription and NF-κB activation.

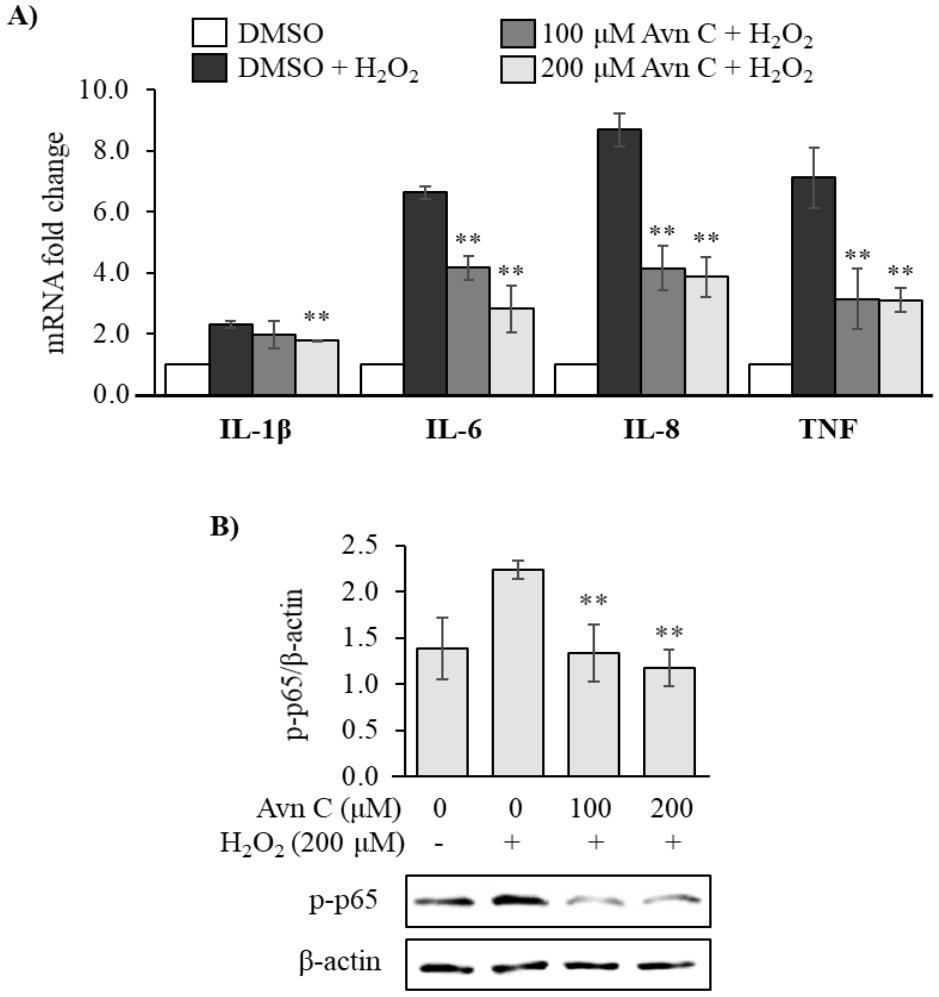


Figure 4.6 Avn C suppresses H_2O_2-induced mRNA expression of pro-inflammatory cytokines and NF-κB activation in 2DD fibroblasts. Normal human skin fibroblasts (2DD) were pre-treated with DMSO (0) or Avn C (100 μM and 200 μM) for 48 h followed by 1 h exposure to 200 μM H_2O_2. A) RT-qPCR on cDNA libraries was performed for *interlukin-1 beta* (*IL-1β*), *interlukin-6* (*IL-6*), *interlukin-8* (*IL-8*) and *tumor necrosis factor (TNF)* gene transcripts. Data are represented as the fold change against control. B) Western blot analysis of phospho-NF-κB p65 (Ser536) (p-p65) expression. Data are presented as a ratio of p-p65 to β-actin. Representative blot images are shown. All graphs indicate mean values from three biological replicates. Error bars represent standard deviations. ***p*-value <0.01 vs H_2O_2 treatment alone.

4.5 Anti-inflammatory potential of Avn C

4.5.1 Avn C protects 2DD fibroblasts from TNFα-induced inflammation

As we observed a protective effect of Avn C against oxidative stress-induced inflammatory response in 2DD fibroblasts, we investigated if the suppression effect of inflammation could be independent from Avn C free radical scavenging ability. Many proteins and signaling molecules can induce a significant stress to activate pro-inflammatory markers. One such molecule is TNF-α, which can either function as an autocrine or paracrine stimulator and triggers signaling events, leading to NF-κB activation in different cell types. Hence, we evaluated if Avn C also could protect 2DD fibroblasts against TNFα-induced inflammation. RT-qPCR results demonstrated that TNF-α treatment alone strongly increased the level of transcripts from the *Il-1β, Il-8* and *TNF-α* genes (Figure 4.7 A). Pre-treatment with Avn C at 100 and 200 μM significantly reduced the mRNA levels of *Il-1β, Il-8* and *TNF-α* induced by TNF-α treatment. Western blot analyses of phosphorylated NF-κB p65 (Ser536) demonstrated that TNF-α treatment alone resulted in an increased protein level of phosphorylated p65, which was significantly reduced by 100 and 200 μM Avn C pre-treatment (Figure 4.7 B).

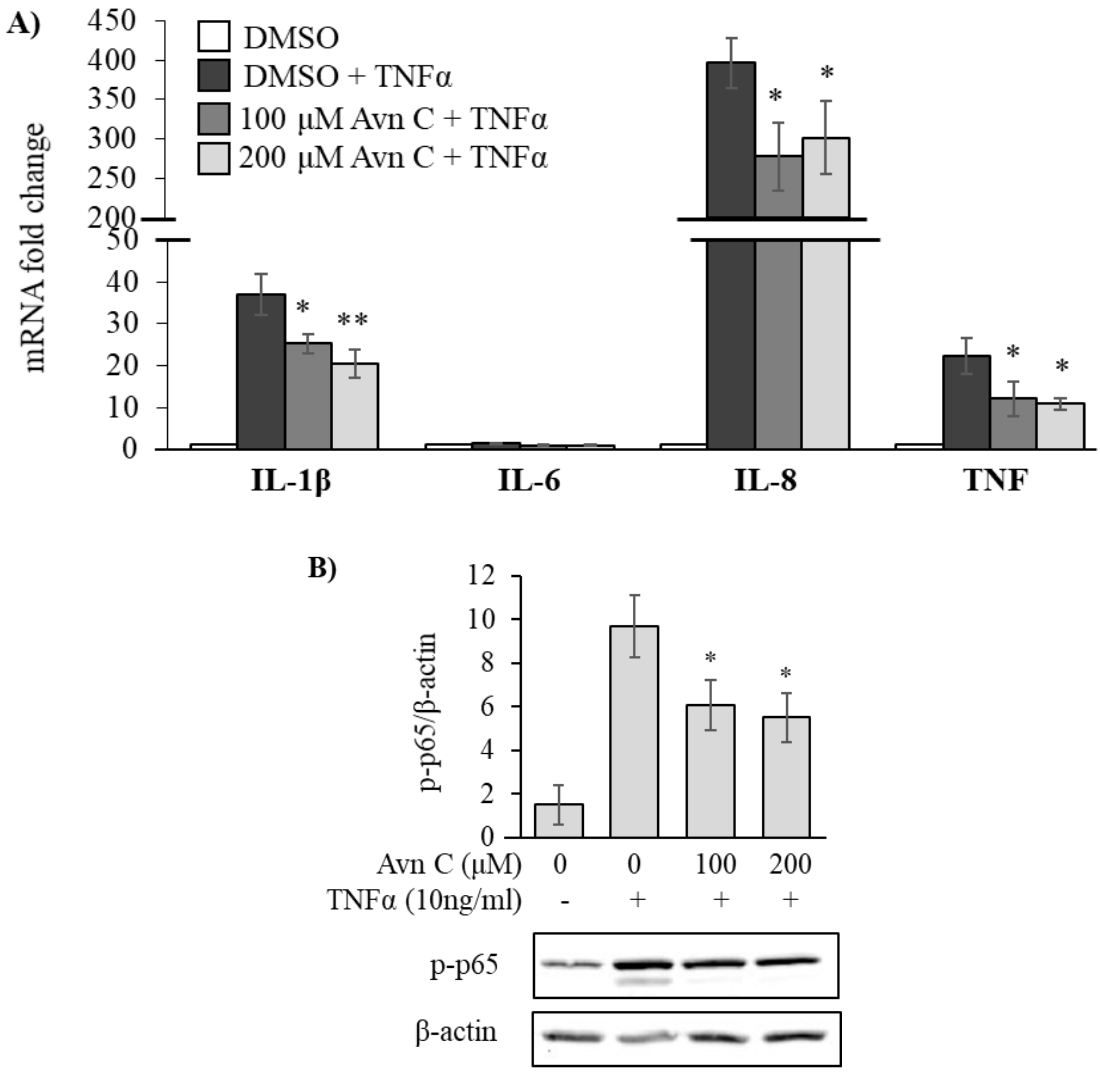


Figure 4.7 Avn C suppresses TNF-α-induced mRNA expression of pro-inflammatory cytokines and NF-κB activation in 2DD fibroblasts. Normal human skin fibroblasts (2DD) were pre-treated with DMSO (0) or Avn C (100 μM and 200 μM) for 24 h followed by 4 hours exposure to 10 ng/mL TNF-α. A) RT-qPCR on cDNA libraries was performed for *interlukin-1 beta* (*IL-1β*), *interlukin-6* (*IL-6*), *interlukin-8* (*IL-8*) and *tumor necrosis factor alpha (TNF)* gene transcripts. Data are represented as the fold change against control. B) Western blot analysis of phospho-NF-*κ*B p65 (Ser536) (p-p65) expression. Data are presented as a ratio of p-p65 to β-actin. Representative blot images are shown. All graphs indicate mean values from three biological replicates. Error bars represent standard deviations. * *p*-value <0.05, ** *p*-value <0.01 vs TNF-α treatment alone.

4.5.2 Avn C reduces basal inflammation through NF-κB inhibition in 2DD fibroblasts

It has been reported and verified by several studies that constitutive low-grade inflammation is tightly associated with the aging process, as supported by NF-κB system activation in various tissues during aging (Spencer *et al.*, 1997; Kim *et al.*, 2002; Kim *et al.*,2006). As we observed that Avn C showed a cytoprotective effect against H_2O_2-induced and TNFα-induced inflammation, we further investigated if Avn C could also exert its anti-inflammatory activity under normal metabolic conditions inside cells. Following Avn C treatment for 48 h on 2DD fibroblasts, RNA was extracted, converted to cDNA and evaluated by RT-qPCR. mRNA fold changes of pro-inflammatory cytokines are shown in Figure 4.8 A. These results demonstrated a significant reduction of *Il-1β, Il-8, IL-6* and *TNF-α* transcripts at the basal level, except for *TNF-α* at 100 μM Avn C treatment. Western blot analyses were performed on cell protein extracts under the same treatment conditions. It was found that 100 and 200 μM Avn C treatment significantly increased protein expression of phosphorylated NF-κB p65 (Ser536) in 2DD fibroblasts (Figure 4.8 B).

To further confirm that Avn C reduced inflammatory response by inhibiting NF-κB activation, chromatin immunoprecipitation-qPCR (ChIP- qPCR) was performed to analyze changes in NF-κB binding activity to promoter regions of *Il-1β* and *Il-8* with 48 h of 100 μM Avn C treatment. We observed that 100 μM Avn C treatment significantly decreased NF-κB binding activity to both *Il-1β* and *Il-8* promoter regions (Figure 4.8 C), indicating that NF-κB activity was down-regulated. Taken together, these findings suggest that Avn C reduces basal inflammation through reduced NF-κB activation in 2DD fibroblasts.

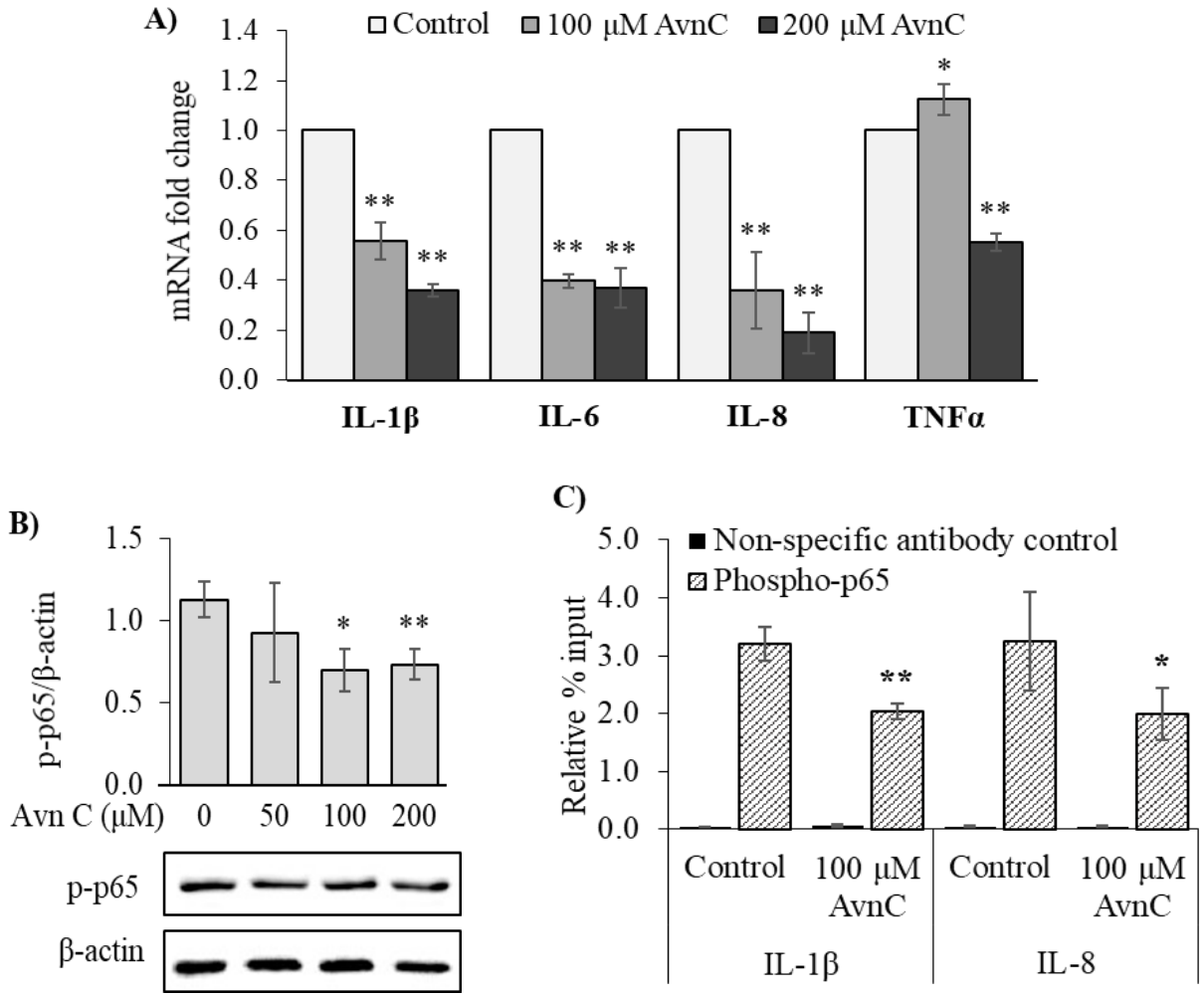


Figure 4.8 Avn C reduces pro-inflammatory cytokine transcriptions through decreased DNA binding activity of NF-κB in 2DD fibroblasts. A) 2DD human skin fibroblasts were treated with DMSO (control) or Avn C (100 μM and 200 μM) for 48 h. RT-qPCR on cDNA libraries was performed for *interlukin-1 beta* (*IL-1β*), *interlukin-6* (*IL-6*), *interlukin-8* (*IL-8*) and *tumor necrosis factor (TNF)* gene transcripts. Data are represented as the fold change against control. B) Western blot analysis of phospho-NF-κB p65 (p-p65) expression in 2DD cells treated with DMSO (0) or Avn C (50 μM, 100 μM and 200 μM) for 48 h. Data are presented as a ratio of p-p65 to β-actin. Representative blot images are shown. C) ChIP-qPCR analysis of phospho-NF-κB p65 binding activity to promoter regions of *interlukin-1 beta* (*IL-1β*) and *interlukin-8* (*IL-8*) in 2DD cells treated with control (DMSO) or 100 μM Avn C for 48 h. Data are represented as the enrichment relative to % input. All graphs indicate mean values from three biological replicates. Error bars represent standard deviations. **p*-value<0.05, ***p*-value <0.01 vs control.

4.6 Avn C induces HO-1 expression through Nrf2 activation in 2DD fibroblasts

Given that Avn C showed a cytoprotective effect against H_2O_2-induced oxidative stress through free radical scavenging which decreased H_2O_2-induced antioxidant gene transcription, we further investigated if Avn C could also exert protective effects by activating other cytoprotective pathways independent of free radical scavenging. 2DD cells were treated with Avn C for 48 h and RNA was extracted, converted to cDNA and evaluated by RT-qPCR to examine if there was any antioxidant enzyme that was up-regulated by Avn C treatment. Transcript quantification by qPCR demonstrated that *HMOX1* was significantly up-regulated by 50, 100 and 200 μM Avn C treatment (Figure 4.9 A), whereas other antioxidant genes either showed no significant change or significant decreases. To further confirm if *HMOX1* gene expression was induced by Avn C treatment, western blot was used to analyze protein expression levels of HO-1, which is coded by the *HMOX1* gene. It was found that 100 and 200 μM Avn C treatment significantly increased protein expression of HO-1 in 2DD fibroblasts (Figure 4.9 B).

Expression of HO-1 is regulated by nuclear factor-E2-related factor 2 (Nrf2) (Itoh *et al.*, 1997; Alam *et al.*, 1999). To investigate the impact of Avn C treatment on Nrf2-DNA binding activity of, 2DD cells were treated with vehicle (DMSO) or 100 μM Avn C for 48 h. Cells were then cross-linked and chromatin immunoprecipitation-qPCR (ChIP- qPCR) was performed to analyze Nrf2 binding activity to promoter regions of *HMOX1* and *NAD(P)H quinone dehydrogenase 1 (NQO1)* genes, which have been identified as Nrf2-regulated genes (Alam *et al.*, 2000; Nioi *et al.*, 2003). Treatment of cells with 100 μM Avn C treatment significantly increased Nrf2 binding activity to both *HMOX1* and *NQO1* promoter regions (Figure 4.9 C). Taken together, these findings indicate that Avn C induced *HO-1* expression through increased DNA binding activity of Nrf2 in 2DD fibroblasts. This indicates that Avn C not only reduces oxidative stress through scavenging free radicals, it could also mediate signaling pathways resulting in cytoprotection.

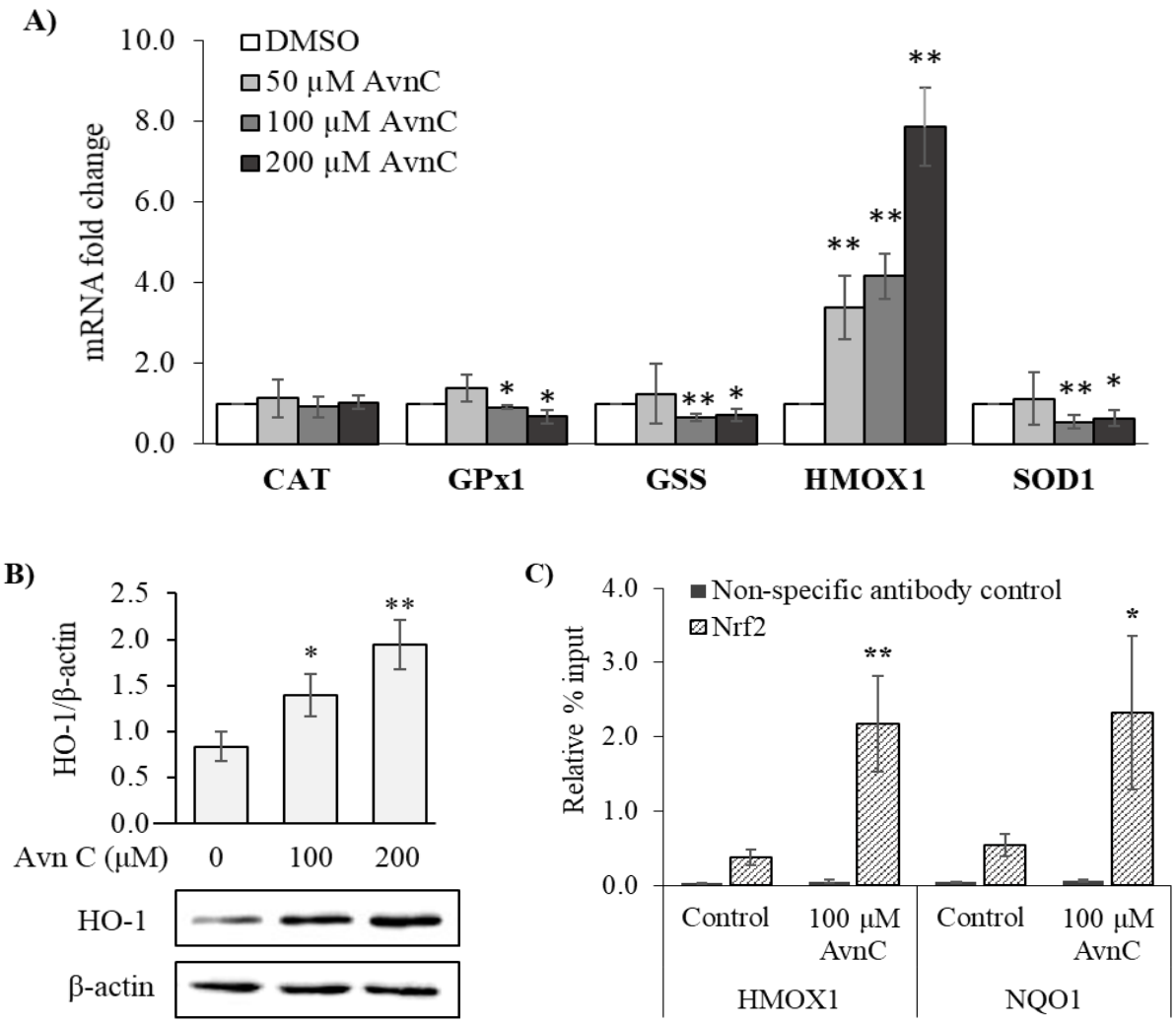


Figure 4.9 Avn C induces heme oxygense-1 expression through increased DNA binding activity of Nrf2 in 2DD fibroblasts. A) 2DD human skin fibroblasts were treated with DMSO (control) or Avn C (50 μM, 100 μM and 200 μM) for 48 h. qRT-PCR on cDNA libraries was performed for *glutathione synthetase* (*GSS*), *heme oxygenase 1* (*HMOX1*), *superoxide dismutase 1* (*SOD1*), *glutathione superoxidase 1* (*GPx1*) and *catalase* (*CAT*) gene transcripts. Data are represented as fold changes against control. B) Western blot analysis of heme oxygenese-1 (HO-1) expression in 2DD cells treated with DMSO (control) or Avn C (100 μM and 200 μM) for 48 h. Data are presented as a ratio of HO-1 to β-actin. Representative blot images are shown. C) ChIP-qPCR analysis of Nrf2 binding activity to promoter regions of *heme oxygenase 1* (*HMOX1*) and *NAD(P)H quinone dehydrogenase 1 (NQO1)* in 2DD cells treated with DMSO (control) or 100 μM Avn C for 48 h. Data are represented as the enrichment relative to % input. All graphs indicate the average values from three biological replicates. Error bars represent the standard deviations. **p*-value<0.05, ***p*-value<0.01 vs control.

4.7 Anti-proliferative effect of Avn C

4.7.1 Avn C reduces proliferative rate of 2DD fibroblasts through autophagy-independent pathway

Results from this research showed that Avn C had a cytoprotective effect against oxidative and inflammatory stress, and also showed anti-aging potential through the activation of Nrf2 and inhibition of NF-κB activity. Studies have demonstrated that select phenolic compounds showed an anti-proliferative effect by activating the autophagy pathway, which promoted cellular health and increased longevity (Baur *et al.*, 2006; Park *et al.*, 2016; Holczer *et al.*, 2018); therefore, we evaluated if Avn C could promote cellular health through similar mechanisms. To determine the anti-proliferative effect of Avn C on 2DD fibroblasts, we treated cells with either vehicle (DMSO) or 50, 100 and 200 μM Avn C for 48 h and monitored their growth. Avn C had a significant impact on proliferative rates in 2DD fibroblasts at 50, 100 and 200 μM concentrations (Figure 4.10 A). Trypan blue staining of cells indicated that there was no significant cell death in any of the treatment conditions used (Figure 4.10 B). To further confirm this response, we immuno-labeled Avn C treated 2DD fibroblasts for the proliferative marker Ki67, which is a nucleolar and chromatin-associated protein that is only present in actively dividing cells. 2DD cells showed a significant decreased in the number of Ki 67 positive cells at 50, 100 and 200 μM Avn C treatment (Figure 4.10 C). These observations indicated that Avn C was modulating cell growth in primary fibroblasts without causing cell death.

As we observed a significantly decrease of proliferative rate with Avn C treated normal human fibroblast cells, we investigated if this anti-proliferative effect was caused by activation of the autophagy pathway. Western blot analyses were performed, and we observed no change in the levels of the autophagy marker protein light chain 3 isoform II (LC3-II) (Figure 4.11). Sirtuin 1 (SIRT1) and mTOR are upstream molecules of autophagy activation and are responsible for sensing cellular energy levels. Western blot results also demonstrated that there was no change in SIRT1 and phospho-mTOR (activated mTOR) protein levels following 48 h Avn C treatment (Figure 4.10). To further confirm Avn C that does not activate the autophagy in normal human fibroblasts, a second normal human skin fibroblast cell line (FSF cells) was employed and showed the same western blot results as 2DD fibroblasts (Figure 4.11), indicating Avn C reduces the proliferative rate of normal human skin fibroblasts through an autophagy-independent pathway.

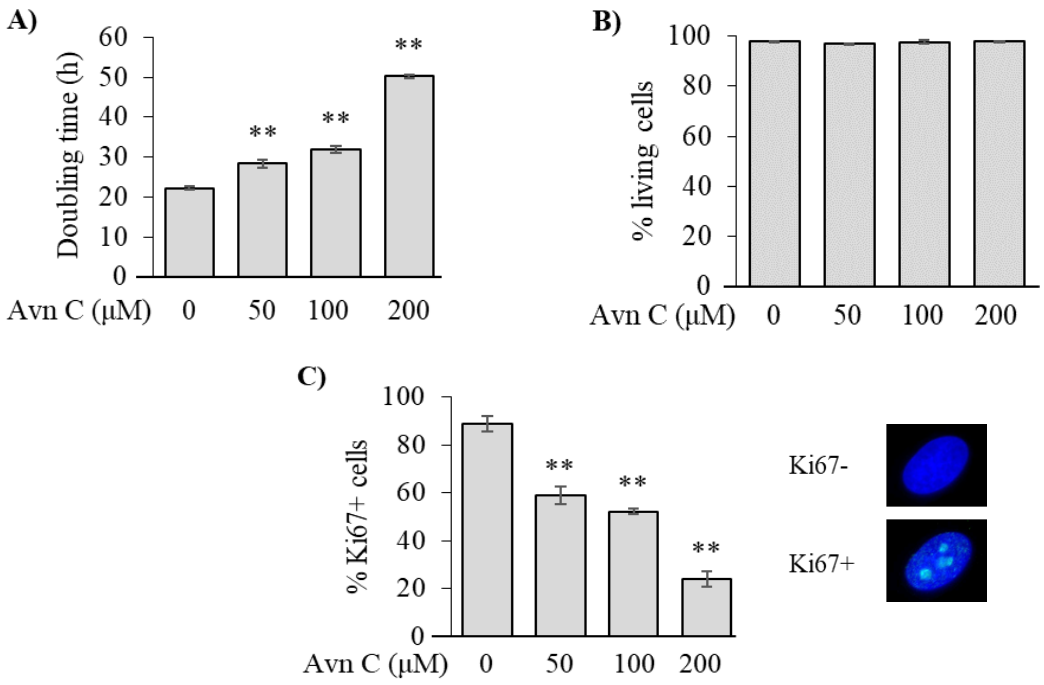


Figure 4.10 Avn C reduces proliferative rate of 2DD fibroblasts without inducing cell death. 2DD fibroblasts were treated with DMSO (0) or Avn C (50 μM, 100 μM and 200 μM) for 48 h. A) Population doubling times. B) Trypan blue assays were conducted to detect percent (%) viable cells. C) Ki67 immuno-labelling of 2DD cells. Percent positive Ki67 cells was shown on the Y-axis. All graphs indicate mean values from three biological replicates. Error bars represent standard deviations. **p*-value<0.05, ***p*-value <0.01 vs control.

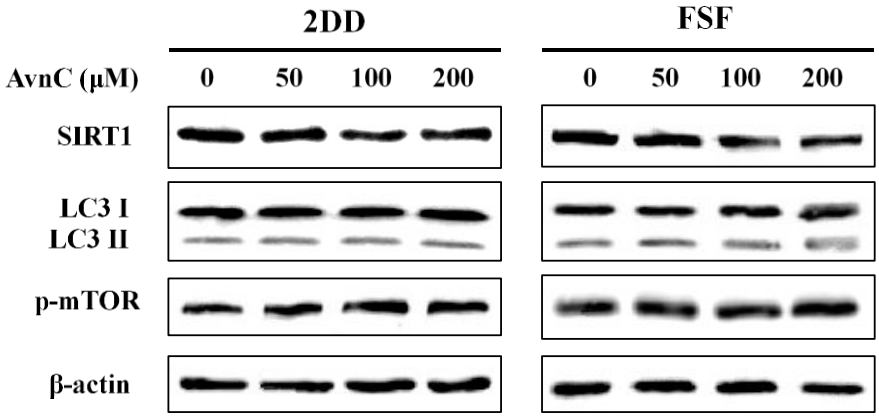


Figure 4.11 Avn C does not induce autophagy in 2DD and FSF primary fibroblasts. Two primary fibroblasts (2DD and FSF) were treated with DMSO (0) or Avn C (100 μM and 200 μM) for 48 h. Whole cell lysates were extracted and subjected to western blot analysis for LC3-II, SIRT1 and phospho-mTOR (p-mTOR). β-actin was used as a loading control

4.7.2 Avn C reduces proliferative rates of breast cancer (MCF7) and bone cancer (U2OS) cells

To determine if Avn C can inhibit the growth of cancer cells, several cancer cell lines, MCF7 (breast), HTC116 (colon), HepG2 (liver) and U2OS (bone) were used in this study. We treated the four cancer cells with vehicle (DMSO) or 50, 100 and 200 μM Avn C for 48 h followed by the determination of population doubling times. Both MCF7 and U2OS cells demonstrated an increase in population doubling times at 100 and 200 μM while HCT116 and HepG2 cells exhibited no change (Figure 4.12). Trypan blue staining of the four different cancer cells treated with Avn C indicated that there was no significant death observed at the concentrations used (Figure 4.12). These observations indicate that Avn C does not promote cell death and may not be effective at killing cancer cells but does have the potential to slow growth in MCF7 and U2OS cells at concentrations of 100 and 200 μM.

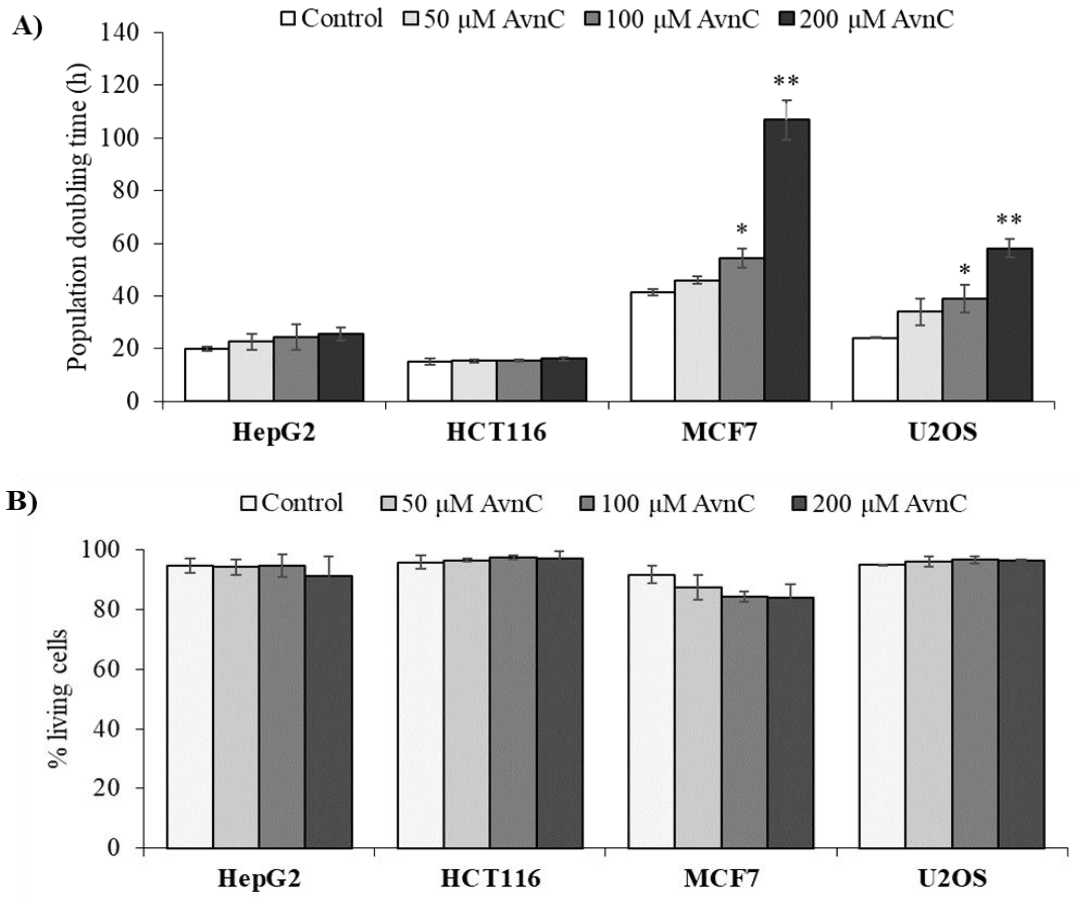


Figure 4.12 Avn C reduces proliferative rates of breast cancer (MCF7) and bone cancer (U2OS) cells without inducing cellular death. MCF-7 cells (human breast adenocarcinoma), HCT 116 cells (human colon carcinoma), HepG2 cells (human liver hepatocellular carcinoma) and U2OS cells

(human bone osteosarcoma) were treated with DMSO (0) or Avn C (50 μM, 100 μM and 200 μM) for 48 h. A) Population doubling times of the four cancer cells treated with DMSO or Avn C for 48h. B) Trypan blue assays were conducted to detect percent (%) viable cells for cancer cells treated with DMSO or Avn C for 48h.

5. DISCUSSION

5.1 Overall discussion

Oat consumption in the human diet has increased in the last decade due to growing interest in the health aspects of oats and oat containing products. Extensive research has focused on the high dietary fibre contents of oats, especially β-glucan, which was found to lower cholesterol levels and provide cardiovascular protection (Martínez-Villaluenga and Peñas, 2017). Recently, avenanthramides, a group of phenolic compounds found exclusively in oats, have received great interest and are considered as important contributors to the health benefits of oats. Avenanthramides are a group of phenolic compounds and structurally shown as amides with different hydroxycinnamic acids and different anthranilic acids (Collin, 1989). Since the discovery of this group of novel phenolic compounds in oats in 1989 (Collin, 1989), several studies have focused on chemically synthesizing avenanthramides and investigating *in vitro* antioxidant activities of Avn A, B and C, which are the three most abundant avenanthramides in oats (Dimberg *et al.*, 1993; Peterson *et al.*, 2002; Lee-Manion *et al.*, 2009; Yang *et al.*, 2014). Despite the use of different antioxidant activity assays, they all showed that Avn C had the highest *in vitro* antioxidant activity among the three. More recent research on the anti-inflammatory and anti-proliferative effects of Avn C or Avn C-enriched oat extracts employing different cell culture models has been conducted (Guo *et al.*, 2008; Sur *et al.*, 2008; Chu *et al.*, 2013; Yang *et al.*, 2014; Nie *et al.*, 2006a; 2006b). In addition, studies evaluating the bioavailability of Avns or oat phenolic-rich extracts in mice and humans found that they were bioavailable at nanomolar-level concentrations in plasma (Chen *et al.*, 2004; Chen *et al.*, 2007). Furthermore, it has been shown that Avn C was extensively metabolized by mice and the human microbiota to generate bioactive metabolites (Wang *et al.*, 2014). When taken together, this scientific evidence shows that Avn C is a compound of great research value with respect to its potential health benefits and mechanisms of action.

Although several studies have shown that Avn C has significant free radical scavenging activity (Peterson *et al.*, 2002; Bratt *et al.*, 2003; Lee-Manion *et al.*, 2009), no study has addressed the antioxidant efficacy of Avn C inside human cells. Additionally, current cellular studies on Avn C treatment has mostly focused on therapeutic effect instead of preventive effect. Furthermore, the mechanisms of Avn C that function as signaling mediators inside cells are still poorly understood. More importantly, the cytoprotective effect of a chemical compound is usually achieved by multiple mechanisms working together such as antioxidant, anti-inflammatory, antigenotoxic and

antiproliferation, through parallel or overlapping cellular pathways and results in an overall promoted cellular health; which has been found for resveratrol and EGCG (Aggarwal and Shishodia, 2006; Visioli *et al.*, 2011; Zhang, 2015). Current research evidence on the cytoprotective effect of Avn C is mainly based on one or two effects in a specific cell line and lacks a comprehensive understanding of possible multiple functions/mechanisms. To investigate the cytoprotective potential of Avn C and better understand the mechanisms of action of Avns in oats, phenolic extracts from ten different oat varieties were produced and the Avns in these extracts were identified and quantified. *In vitro* radical-scavenging assays were used to confirm that Avn C had the highest antioxidant potential among the three Avns. In addition, multiple cytoprotective effects of Avn C on normal human skin cells were explored including antioxidant, antigenotoxicity, anti-inflammation and antiproliferation. Also, several potential molecular targets of Avn C that might attribute to its cytoprotective effects were investigated. Moreover, four cancer cell lines from different organs were employed for investigating the possible anticarcinogenesis effect of Avn C.

In this research, Avn A, B and C in oat extracts were identified and quantified by HPLC-PDA. Results showed that different oat vareties or lines had significant difference with regard to concentrations of Avn A, B & C, total phenolic content and free radical scavenging activity in oat extracts. *In vitro* radical-scavenging results showed that Avn C not only had the highest antioxidant activity among the three avenanthramides, it also showed higher antioxidant activity than caffeic acid and Trolox, which are commonly considered as strong antioxidants. From my cellular studies, I demonstrated for the first time that Avn C protected human skin fibroblasts from H_2O_2-induced cellular damage as shown by significantly reduced levels of intracellular free radicals, DNA damage and pro-inflammatory response. Pre-treatment of Avn C also protected cells against signaling to promote inflammatory responses induced by TNF-α, indicating its functional role as a signaling mediator other than free radical scavenger. Furthermore, mechanistic studies revealed that Avn C suppressed basal level of inflammation through decreased NF-κB DNA binding activity and induced heme oxygenase 1 expression through increased Nrf2 DNA binding activity, indicating that Avn C exerts antioxidant and anti-inflammatory effect, either or indirectly through modulating NF-κB and HO-1/Nrf2 signaling pathways apart from free radical scavenging. In addition, Avn C slowed the growth rate of normal human skin fibroblasts through autophagy-

independent mechanisms. It also suppressed the growth of human breast cancer cells and bone cancer cells but did not induce cell death.

5.2 Identification and quantification of Avn A, B and C in oat seeds from ten varieties and breeding lines

Previous studies reported that Avn A, B and C are the most abundant avenanthramides in oats, whereas other identified avenanthramides have only been found in trace amount (Collins, 1989; Bratt *et al.*, 2003; Peterson and Dimberg, 2008); therefore, in this research, we focused on identifying and quantifying the three major avenanthramides from oat seeds of ten varieties and lines grown in Saskatoon, Saskatchewan, Canada. Phenolics were released from oat powder by treatment with 80% methanol to produce a phenolic-rich extract. The concentrated phenolic-rich extracts were then subjected to phenolic analysis using a reversed-phase HPLC-PDA method. The extraction and HPLC methods used in this research were adapted from those reported in literature, where phenolic separation and quantitation was obtained (Dimberg *et al.*, 1993; Chu *et al.*, 2013; Ishihara *et al.*, 2014). Chemically synthesized Avn A, B and C were used as standards for both identification and quantitation in these phenolic-rich extracts. Based on chromatographic evidence, all ten oat extracts showed clear and sharp peaks that were identified as Avn A, B and C, which indicates these phenolics were effectively extracted and identified using the methods described. Concentrations of the identified Avn A, B and C in the ten phenolic-rich extracts were determined from linear calibration equations of Avn standards (concentration vs. peak area). Different oat varieties showed large concentration differences of the three avenanthramides (Table 4.1). The highest concentrations of individual and total Avn A, B and C were all found in BW10, followed by CDC Dancer and OT2021. Several studies have reported concentrations of the three avenanthramides from oat seeds grown in different countries. One study found that the concentration ranges of Avn A, B and C in three Swedish oat cultivars as 25-47 mg/kg, 21-43 mg/kg and 21-43 mg/kg, respectively (Dimberg *et al.*, 1996). Another study tested four oat cultivars in U.S.A and found that the average concentrations of Avn A, B and C were 54 mg/kg, 36 mg/kg and 52 mg/kg, respectively (Emmons and Peterson, 1999). The difference between literature values and the values obtained in this study are most likely due to different genotype effects and environmental factors. Notably, Chu and colleagues identified and quantified Avn A, B and C from seven common Canadian oat varieties including CDC Dancer and CDC Morrison

(Chu *et al.*, 2013), which were the same two varieties as we tested in this research. They found that CDC Dancer contained 5.06 mg/kg Avn A, 7.96 mg/kg Avn B and 7.15 mg/kg Avn C; CDC Morrison contained 1.05 mg/kg Avn A, 3.66 mg/kg Avn B and 3.70 mg/kg Avn C (Chu *et al.*, 2013). These values of the two oat varieties were around 2-4 times lower than the analytical results obtained in this study. It could be explained by the influence of environmental factors such as light, temperature and agronomic practices on oat cultivars. Furthermore, this indicates that Canadian grown cultivars are excellent candidates for extraction of avenanthramides for nutraceutical use.

5.3 *In vitro* antioxidant activity of oat extracts and Avn A, B & C

Several *in vitro* antioxidant activity assays are commonly used in research where the antioxidant potential of phenolic-rich extracts and pure compounds are determined, as they are relatively quick and convenient. The most commonly used methods are the ABTS and DPPH radical scavenging assays, ferric reducing ability of plasma (FRAP) assay, oxygen radical absorbance capacity and the inhibition of β-carotene bleaching (Prior *et al.*, 2005). In this study, we used the ABTS and DPPH *in vitro* methods to evaluate the antioxidant activity of oat extract samples and pure avenanthramides.

Initially, the total phenolic contents of oat phenolic-rich extracts were determined by the Folin-Ciocalteu method. It was found that total phenolic content of the ten phenolic-rich extracts ranged from 78.5 ± 0.7 to 121.2 ± 2.5 mg GAE/100 g oat. The highest total phenolic content was found in BW10, followed by OT 2021 and BW5303. In literature, Chu and colleagues used the same method to determine total phenolic contents of seven oat varieties. They found that the range of total phenolic content was 57 to 94 mg GAE/100 g (Chu *et al.*, 2013), which was lower than what was found in this study. In addition, total phenolic content of CDC Dancer and CDC Morrison were reported as 62 and 63 mg GAE/100 g in their study (Chu *et al.*, 2013), which were also lower than the values we obtained. These differences in TPC values could be explained by the different extraction method used and/or chemical compound compositional differences impacted by environmental factors such as light, temperature and agronomic practices. Environmental factors are known to influence the expression of genes through modifying epigenetic profiles. Further analyses of these cultivars from these environmental conditions could identify the up-regulation of genes involved with the biosynthesis of Avns leading to increased levels.

Furthermore, ABTS and DPPH radical scavenging activities of the ten oat phenolic-rich extracts were measured. The highest ABTS radical scavenging activity was found for BW10, followed by OT2021 and CDC Dancer. The highest DPPH radical scavenging activity was found in OT2021, followed by BW10 and CDC Dancer. In literature, several studies have reported ABTS and DPPH radical scavenging activities of oat extracts arising from different parts of oat seeds (e.g. brans and hulls). The methods they used were unstandardized, which leads to the use of different reaction times, temperatures, reference standards and expressed units; therefore, it is difficult to compare our results to literature values. However, taken together the results from three different *in vitro* antioxidant assays used in this study, a consistent conclusion can be obtained, which is that oat phenolic-rich extracts from BW10 and OT2021 have the highest antioxidant activity among the ten oat varieties and lines. However, we found that CDC Dancer extract had the higher total concentrations of Avn A, B and C than OT2021 while OT2021 extract had the higher antioxidant than CDC Dancer. The reason might be that other phenolic compounds in oat extract also play critical roles in free radical scavenging and they may have synergistic effects.

In addition to oat phenolic-rich extracts, the *in vitro* antioxidant activities of pure Avn A, B and C were also determined. It was observed that Avn C not only showed the highest ABTS and DPPH radical scavenging activities among the three avenanthramides, it was also higher than caffeic acid and Trolox, which are commonly considered as strong antioxidants. It could be explained by the differences in the number of phenol structure. Avn C has three phenol structures while Avn A, Avn B, Trolox and caffeic acid have less than two phenol structures. Similar results can be also found in literature. One study showed that Avn C had higher antioxidant activity than Avn A and Avn B using DPPH assay and β-carotene bleaching assay (Peterson *et al.*, 2002). They also found that Avn C had more than twice DPPH radical scavenging activity than Trolox (Peterson *et al.*, 2002). Another study used DPPH assay and ferric reducing ability of plasma (FRAP) assay to demonstrate that Avn C not only showed highest antioxidant activity among the three avenanthramides, it also had higher antioxidant activity than caffeic acid (Lee-Manion *et al.*, 2009). Taken together the results from our study and literature, we can see Avn C has strong antioxidant potential.

5.4 Protective effect of Avn C against cellular stress

Although *in vitro* antioxidant activity results showed high antioxidant potential of Avn C, it is unclear if this compound can effectively pass through cells and exert cytoprotective effects. Therefore, we used normal human skin fibroblast cells (2DD cell line) to investigate the protective effect of Avn C against cellular stress including oxidative stress and inflammatory stress. In order to evaluate disease preventive potential instead of therapeutic potential of Avn C, 2DD cells were pre-treated with Avn C for 48 h and then stimuli were applied to induce cellular stress. Following treatment, several assays were employed to determine and confirm if Avn C had the ability to protect 2DD cells from induced cellular damage.

Initially, the protective effects of Avn C against H_2O_2-induced oxidative stress were investigated. It has been proposed that cellular damage induced by oxidative stress is mostly associated with the production of H_2O_2, because it is more stable when compared with hydroxyl radicals or superoxide anions, and more capable of diffusing throughout a larger tissue area (Yuan *et al.*, 2003; Ohshima, 2004). In order to evaluate oxidative stress levels inside cells, we used Mitotracker Orange dye to label free radicals inside cells. Mitotracker Orange is a non-fluorescent dye in its reduced form. When it interacts with free radicals, it is oxidized and shows orange fluorescence. By observing fluorescence intensity, we found that pre-treatment with Avn C reduced intracellular free radical levels induced by H_2O_2, thus indicating that Avn C was not only able to penetrate cells but also reduced intracellular free radical levels. It indicates that Avn C may directly neutralize free radicals inside cytoplasm before cellular components being damaged by free radicals. Furthermore, transcriptional levels of antioxidant enzymes were measured. Antioxidant enzymes are part of our endogenous antioxidant defense system and their expressions are usually stimulated by free radicals (Matés *et al.*, 1999); therefore, they are also indicators of oxidative stress. We found that Avn C exposure lead to significant decreased levels of antioxidant enzyme transcripts induced by H_2O_2, which confirmed the intracellular antioxidant effect of Avn C.

We then investigated the protective effects of Avn C against H_2O_2-induced inflammatory responses and DNA damage. Functional roles of H_2O_2 in inflammation have been investigated by several studies. Results have shown that H_2O_2 can act as a signaling molecule that activates inflammatory response through phosphorylation of tyrosine residues in NF-κB and its upstream regulators such as IκB and IKK (Schoonbroodt *et al.*, 2000; Wittmann *et al.*, 2012). In addition,

cellular damage caused by H_2O_2 releases TNF-α from damaged cells, resulting in the amplification of pro-inflammatory stimuli (Kim *et al.*, 2008). In this study, it was observed that H_2O_2 induced the transcriptional expressions of *Il-1β, Il-8, IL-6* and *TNF-α*, which were significantly reduced by Avn C pre-treatment. It was also found that H_2O_2 induced the expression of phosphorylated p65 (p-p65), which is usually used as a marker for the activation of NF-κB and increased inflammatory response. Pre-treatment with Avn C significantly reduced the levels of p-p65 induced by H_2O_2. These observations indicated that Avn C has the ability to protect cells against free radical induced inflammatory responses. In addition to inflammation, DNA damage is another major effect of free radicals. Increased levels of DNA damage results from increased levels of free radicals and results in increase mutation rates, leading to cancer and a variety of age-related disorders (Wallace, 2002; Evans *et al.*, 2004). Our results demonstrated that pre-treatment with Avn C significantly reduced H_2O_2-induced protein expression of *p*-H2AXγ, which is a sensitive marker for DNA double-strand breaks (Mah *et al.*, 2010). Our observations demonstrate that Avn C has the ability to protect cells against free radical induced DNA damage, resulting in an increased DNA stability and function. This on its own, is a positive cytoprotection effect which would lead to increased cellular health.

Given that Avn C was shown to have the ability to protect 2DD cells against oxidative stress-induced inflammatory response, further investigation was conducted to determine if Avn C can also protect cells from TNF-α induced inflammation. TNF-α was used instead of H_2O_2 to induce a pro-inflammatory response inside 2DD cells. TNF-α can either act as an autocrine or paracrine stimulator, binds cell receptors and trigger signaling events, leading to NF-*κ*B activation and pro-inflammatory cytokines expression (Liu and Han, 2001). Results from this study showed that TNF-α induced the transcriptional levels of *Il-1β, Il-8* and *TNF-α*, which were significantly reduced by Avn C pre-treatment. Notably, only a slight increase in *Il-6* transcripts was observed with TNF-α treatment, indicating that H_2O_2 and TNF-α have different impacts on pro-inflammatory markers in primary skin fibroblasts. Pre-treatment with Avn C also showed a mild but significant decrease in the protein levels of p-p65 induced by TNF-α. These results suggest that Avn not only acts as radical scavenger for cellular protection, it can also function as a signaling mediator to regulate inflammatory response.

5.5 Impact of Avn C on gene regulation

As Avn C was shown as an effective free radical scavenger that can protect cells against H_2O_2-induced and TNFα-induced cellular damage, including oxidative stress and inflammatory response, we further investigated if Avn C had an impact on gene expression that might attribute to its protective effects. We used the same cell line 2DD cells and treated them with either DMSO or AvnC for 48 h without any stress induction.

As we observed anti-inflammatory effect of Avn C against H_2O_2-induced and TNF-α-induced pro-inflammatory responses, we further investigated if Avn C could also reduce the basal level of inflammation through NF-κB inhibition. Several studies indicate that chronic low-grade inflammation is tightly associated with the aging process, including activation of the NF-κB system in various tissues (Spencer *et al.*, 1997; Kim *et al.*, 2002; Kim *et al.*,2006). It has also been shown that the expression of several inflammatory cytokines was increased during aging and they could be used as reliable aging parameters, including IL-6, TNF-α and IL-1β (Johnson, 2006; Salminen *et al.*, 2008). Our results demonstrated that Avn C reduced basal inflammation through reduced pro-inflammatory cytokine transcripts, phosphorylated NF-κB expression and NF-κB DNA binding activity. The latter result was contradictory to a previous study that suggested that Avns do not directly inhibit the binding of NF-κB to DNA (Guo *et al.*, 2008). However, we used a ChIP assay to directly show a decreased binding activity of NF-κB to DNA. These results demonstrate the suppression effect of Avn C on basal low-grade inflammation through NF-κB inhibition, indicating its potential as an anti-aging strategy.

It has been found that many polyphenols (e.g. resveratrol and EGCG) influence cellular pathways that up-regulate antioxidant enzymes, which are involved in their antioxidant mechanisms (Khan *et al.*, 1992; Lee *et al.*, 1995; Khan *et al.*, 2013). Experiments were conducted to evaluate if Avn C had a similar impact on antioxidant enzymes as resveratrol and EGCG. From the results, *SOD1, CAT, GPx* and *GSS* transcripts were not up-regulated by Avn C treatment; however, a significant increase in HO-1 expression was found. Cumulative evidence has shown that HO-1 is one of the most critical cytoprotective mechanisms due to its anti-oxidant, anti-inflammatory and anti-apoptotic effects (Morse and Choi, 2005). HO-1 is a highly inducible form of heme oxygenase, which catabolizes heme to produce biliverdin, Fe^{2+}, and CO (Maines, 1997). The expression of HO-1 is regulated by its transcription factor Nrf2. Under non-stress conditions, Nrf2 is sequestered in the cytoplasm by the actin-binding protein, Keap1. Following Keap1

degradation, Nrf2 is translocated from the cytosol to the nucleus to induce gene expression by binding to ARE and drive the expression of antioxidant enzymes (Itoh *et al.*, 1999). In a previous study, Fu and colleagues found that Avn C induced HO-1 expression through increased Nrf2 translocation in immortalized kidney epithelial cells (Fu *et al.*, 2015). Here, we demonstrated that Avn C up-regulated HO-1 expression in normal human fibroblasts and further determined an increased Nrf2 DNA binding activity employing a ChIP assay. These results revealed that Avn C provides cytoprotection via mechanisms other than free radical scavenging and NF-κB inhibition. Moreover, activation of Nrf2 in response to Avn C indicates a novel role for polyphenol in promoting cellular health and further indicates that Avn C could be used as a nutraceutical to promote increased healthy lifespan.

As we observed a significantly decrease of proliferative rate with Avn C treated normal human fibroblast cells, we investigated if this effect was caused by activation of the autophagy pathway. Some promising polyphenols such as resveratrol and EGCG showed anti-proliferative and cytoprotective effects through activation of autophagy, which was mediated by SIRT1 activation and mTOR inhibition (Park *et al.*, 2016; Holczer *et al.*,2018). Autophagy is a regulated destructive mechanism of the cell that functions by protein degradation and turnover of the destroyed cell organelles for new cell formation (Morselli *et al.*, 2009). This mechanism is one of the most critical strategies for cellular protection and strongly associated with healthy lifespan extension (Nakamura and Yoshimori, 2018). However, Avn C treated 2DD and FSF cells did not show changes in the expression of the autophagy marker protein LC3-II. Also, SIRT1 and phosphorylated mTOR, which are upstream molecules of autophagy activation and responsible for sensing cellular energy levels, did not show any changes either. These results indicate that Avn C reduces proliferative rate of normal human skin fibroblasts through autophagy-independent pathway. Although Avn C does not activate autophagy in human skin fibroblasts, it still shows the ability to slow down the growth rate of cells without causing cell death. Reduced growth rate represents reduced metabolic rate and reduced level of free radical generation, which might attribute to another possible mechanism of action for promoting cellular health. In addition to normal skin fibroblasts, Avn C also slowed down the growth rates of two cancer cell lines, which were MCF-7 (breast cancer cells) and U2OS (bone cancer cells). Although no significant cell death was found with AvnC-treated cancer cells, which shows that Avn C may be usable as adjunct treatments in combination with other chemotherapeutic agents such as taxol.

6. CONCLUSIONS

The central hypothesis of this research is that Avn C not only has potential antioxidant activity, it can also activate signaling cascades that regulate gene expression involved in cytoprotection and regulates proliferative rates of normal human cells and cancer cells. To validate our hypothesis, Avn A, B and C from oat seeds of ten varieties and breeding lines were extracted, identified and quantified by HPLC-PDA method. Next, antioxidant activities of oat extracts and Avn A, B & C were evaluated employing ABTS and DPPH free radical scavenging systems. In addition, protective effects of Avn C against cellular damage induced by oxidative stress and inflammation were investigated. Furthermore, the impact of Avn C on gene regulation involved in inflammation and cryoprotection was also investigated. Finally, the impact of Avn C on the proliferation of human normal skin cells and cancer cells was determined.

From results obtained from HPLC-PDA, it was found that different oat varieties showed large concentration differences of the three avenanthramides. The mean total amount of Avn A, B and C in the ten oat extracts ranged from 8.37 to 90.58 mg/kg oat. The concentrations of Avn A, B and C present in ten oat varieties and lines showed ranges of 1.76 to 21.39, 2.15 to 34.67 and 4.43 to 34.52 mg/kg oat, respectively. Among ten oat varieties and lines, BW10 contained the highest amount of total and individual Avn A, B and C. Results from three different *in vitro* antioxidant assays used in this study demonstrated a consistent conclusion, which is that oat extracts from BW10 and OT2021 have the highest antioxidant activity among the ten oat varieties and lines. As for pure avenanthramides, Avn C not only showed the highest *in vitro* antioxidant activity among the three avenanthramides, it also showed higher *in vitro* antioxidant activity than caffeic acid and Trolox.

Cytoprotective effect of Avn C against cellular stress was evaluated in normal human skin fibroblast (2DD cells). We found that Avn C was capable of penetrating into cells and scavenging intracellular free radicals induced by H_2O_2. In addition, pre-treatment with Avn C also protected cells from H_2O_2-induced antioxidant enzyme transcription, pro-inflammatory biomarker expressions and DNA double-strand breaks. Furthermore, pre-treatment with Avn C also reduced inflammatory responses induced by TNFα, indicating Avn C could function independently from its free radical scavenging ability and suppress inflammation.

Mechanistic studies revealed that Avn C suppressed basal level of pro-inflammatory transcripts and phosphorylated p65 through decreased NF-κB DNA binding activity. Moreover, Avn C induced HO-1 expression through increased Nrf2 DNA binding activity. Those results indicate that Avn C exerts antioxidant and anti-inflammatory effect through modulating NF-κB and HO-1/Nrf2 signaling pathways apart from free radical scavenging. In addition, Avn C slowed down the growth rate of normal human skin fibroblasts through autophagy-independent mechanisms. It also suppressed the growth of human breast cancer cells and bone cancer cells but did not induce cell death.

In conclusion, Avn C protected cells from oxidative stress, DNA damage and inflammatory response. These cytoprotective effects were associated with its free radical scavenging activity, pro-inflammatory cytokine suppression, NF-κB inhibition, HO-1 induction and Nrf2 activation. Taken Together, Avn C exhibits multidirectional cytoprotective capabilities that contribute to an overall improvement of cellular health, suggesting its potential in disease prevention and promoting increased healthy lifespan.

7. FUTURE DIRECTIONS

Results from this study illustrated cytoprotective mechanisms of Avn C including free radical scavenging, suppression of NF-κB and activation of HO-1/Nrf2 pathways. More mechanistic studies could be conducted. In order to find candidate targets, RNA-sequencing and ChIP-sequencing in normal human cells treated with Avn C can be performed to provide new insights on other possible and novel cellular pathways and molecular targets of Avn C. Moreover, results from this study showed that Avn C did not induce the expression of SIRT1, which is a major energy sensor inside cells and strongly associated with many cytoprotective pathways. However, it did not indicate if SIRT1 was activated or not. It is possible that although Avn C did not induce SIRT1 expression, but it activated SIRT1 function, which might lead to more cellular pathways influenced by Avn C. Further analyses such as knock out or knock down of SIRT1 could elucidate if Avn C has an impact on SIRT1 activation. In addition, Avn C slowed down the growth rates of 2DD fibroblasts, breast cancer cells and bone cancer cells, but no convincing evidence was found to explain the mechanisms of this anti-proliferative effect. Further studies could focus on the impact of Avn C on expression of proteins involved in cell cycle regulations such as cyclin-dependent kinases (CDKs) and p53 pathways.

As most polyphenols are not very stable and they are likely degraded in cell culture media after a period of time, further research could also be done to investigate half-life of Avn C and its depredated products in culture media. This would have implications on the potential half-life of these compounds in the blood stream. Furthermore, the evidence we obtained in this study was based on cell treatments using concentrations much higher than physiological concentrations. Also, limited evidence was found in literature providing bioavailability of Avn C in human. Phase I and II reactions in the liver clear compounds from the blood and reduce their bioavailability. Therefore, further studies using animal models and human trails could provide more evidence on bioavailability, toxicity, functional roles of Avn C and the fate of its bio-transformed metabolites in physiological systems.

In summary, the future directions of this work could range from more mechanistic studies using cell culture models to the studies of functional roles of Avn C in physiological systems employing animal models and human trails. Those works taken together could provide convincing evidence on the effectiveness of Avn C in disease prevention and human health.

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